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ERRESBERATROLA ETA AZIDO DOKOSAHEXAENOIKO ANTIOXIDATZAILEEN EFEKTU NEUROBABESLEA HIPOXIA-ISKEMIAK ARRATOIETAN ERAGINDAKO BURMUIN-KALTEAN

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NEUROPROTECTIVE EFFECTS OF RESVERATROL AND DOCOSAHEXANOIC ACID ANTIOXIDANTS IN PERINATAL HYPOXIC-ISCHEMIC BRAIN INJURY IN RATS

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Aita eta Amari, Ama eta Aitari

Neretxuri

“Pasioa da hemen exijitzea zilegi den gutxieneko hori”

Berritxarrak

“Egia bakarra da, errorea anitza”

Simone de Beauvoir

“Non bisogna temeré i momenti difficili,
perché è da quelli che viene il meglio”

Rita Levi-Montalcini

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Abuelari, nortasun indartsu hori izateagatik, eta aititeri, oraindik ere faltan botatzen zaituztedalako.

Neretxuri, nire pertsonatxoa izateagatik, mundua serioki ez hartzen laguntzeagatik, irakatsi dizkidazun gauza guztiengatik, ni ulertzeagatik, ni maitatzeagatik.

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Mila esker denoi, bihotzez!

One of the most common causes of mortality and morbidity in children is perinatal hypoxia-ischemia, so new and more effective neuroprotective strategies are urgently required, in order to minimize as much as possible the neurological consequences of this encephalopathy. In this sense, interest has grown in the neuroprotective possibilities of antioxidants, such as resveratrol and docosahexaenoic acid.

Resveratrol is a natural polyphenol found in different plant species, including grapevines, peanuts and pomegranates, with the ability to scavenge a variety of free radicals and reactive oxygen species. Docosahexaenoic acid is a long-chain omega-3 fatty acid, commonly found in fish such as salmon and tuna with anti-oxidant and anti-inflammatory properties that is believed to be neuroprotectant against experimental brain injury.

The aim of the present work is to study the important role of those antioxidants independently and the combination of both as a neuroprotective strategy against perinatal hypoxic-ischemic brain injury in perinatal rats.

Our results indicate that pretreatments with resveratrol and docosahexaenoic acid protect against brain damage, reducing infarct volume, preserving myelination and minimizing the astroglial reactive response and microglial activity. Moreover their neuroprotective effects were found to be long lasting, as behavioral outcomes and neuronal conexions were significantly improved at adulthood. We speculate that one of the mechanisms for their neuroprotection may be related to the maintenance of the mitochondrial inner membrane integrity and potential, and to the reduction of reactive oxygen species.

Umeen erikortasun eta hilkortasunaren kausa nagusienetariko bat hipoxia-iskemia perinatala da, hortaz bere aurkako estrategia neurobabesle berri eta eraginkorragoak aurkitzearen premia, entzefalopatia honen ondorio neurologikoak ahalik eta gehien murriztu ahal izateko. Zentzu honetan erresberatrola eta azido dokosahexaenoikoa bezalako antioxidatzaileen aukera neurobabesleen interesa handitu izan da azken aldi honetan. Erresberatrola zenbait landare espezieek ekoizten duten polifenol naturala da, erradikal askeak eta oxigenoaren espezie erreaktiboak ezabatzeko ahalmena daukana. Azido dokosahexaenoikoa katea luzeko omega-3 gantz azidoa da, izokina eta atuna bezalako arraietan aurki dezakeguna.

Lan honen helburu nagusiena antioxidatzaile hauek banaka eta bien arteko konbinazioak hipoxia-iskemia perinatalak arratoeitan eragindako burmuin-kaltearen aurkako eragin neurobabeslea ikertzea izan zen.

Gure emaitzek iradokitzen dute erresberatrola eta azido dokosahexaenoikoaren pretratamenduek hipoxia-iskemia neonatalak eragindako burmuin-kaltean efektu neurobasbesleak dituela, infartuaren bolumena txikitu, zelulen kaltea leundu, mielinaren ekoizpena mantendu, astrogliosi errekatiboa eta mikrogliaren aktibazioa urritu baitute. Gainera, haien eraginak epe luzera hedatzen dira, animalia helduetan egindako frogen ostean antioxidatzaileek kognizio asaldurak eta konexio neuronalen galera ekiditen dituztelako. Antioxidatzaile hauen mekanismo neurobabesletako bat mitokondriaren barne-mintzeko osotasuna eta trasmintzeko potentziala mantentzea dela espekulatzen dugu, oxigenoaren espezie erreaktiboen ekoizpena gutxitzeaz gain.

ABBREVIATIONS/ LABURDURAK

ABC	Avidin Biotin Complex / Abidin-biotina konplexua
ACPD	1-amino-1,3-cyclopentrane dicarboxylic acid
AIF	Apoptosis Inducing Factor / Apoptosia induzitzen duen faktorea
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	ANalysis Of VAriance / Bariantzaren analisi anitza
Apaf-1	Apoptosis protease-activating factor-1
ATP	Adenosine tri phosphate
BAX	Bcl-2-like protein 4
BBB	Blood-Brain Barrier / Barrera hematoentzefalikoa
BDA	Biotinylated Dextran Amines
BSA	Bovine Serum Albumin
Bcl-2	B-cell lymphoma 2
Ca^{2+}	Calcium ion / Kaltzio ioia
CASP-3	Caspase-3 / Kaspasa-3
CYCS	Cytochrome c / c zitokromoa
COX 2	Cyclooxygenase / Ziklooxigenasa
CNS	Central Nervous System / Nerbio Sistema Zentrala (NSZ)
cDNA	Complementary DeoxyriboNucleic Acid / DNA osagarria
c-FOS	FBJ osteosarcoma oncogen
CO_2	Carbone DiOxide / Karbono dioxidoa
CSPG4	Chondroitin Sulfate Proteoglycan 4
CTX	Cortex /Kortexa, garun-azala
DAB	3,3'-Diaminobenzidine / Diaminobentzidina
DAPI	4',6-diamidino-2-phenylindole

DCFH-DA	2,7-dichlorodihydrofluorescein diacetate
DG	Dentate Gyrus / Hortz zirkunboluzioa
DHA	Docosahexaenoic acid / Azido dokosahexaenoikoa
DMSO	Dimetilsulfoxid
DNA	DeoxyriboNucleic Acid /Azido deoxirribonukleikoa
EGR 1	Early Growth Response 1
eNOS	Endothelial Nitric Oxide Synthase / Oxido nitriko sintasa endoteliala
EPO	Erythropoietin / Eritropoietina
FALLS	Forward Angle Light Scatter
GFAP	Glial Fibrilary Acidic Protein / Gliaren proteina azidiko fibrilarra
GSH	Glutathione/ Glutationa
GPx	Glutathione peroxidase / Glutation peroxidasa
GSSG	Glutathione disulfide / glutation disulfuroa
H^+	Hydrogen ion / Hidrogeno ioia
H_2O	Water / Ura
H_2O_2	Hydrogen peroxide / Ur oxigenatua, Hidrogeno peroxidoa
HBT	Hole Board Test / Plataforma zulatuaren froga
HI	Hypoxia-Ischemia / Hipoxia-iskemia
HIE	Hypoxic-Ischemic Encephalopathy / Entzefalopatia hipoxiko-iskemikoa
HIF1	Hypoxia Inducible Factor 1 / Hipoxiak induzitzen duen 1.go faktorea
HSA	Human Serum Albumin
HSF 1	Heat Shock Factor 1
Iba-1	Ionized calcium binding adaptor molecule 1 /

	Ionizaturiko kaltzioari lotzeko molekula moldakor 1
IL-2	Interleukin-2 / Interleukina-2
IL-6	Interleukin-6 / Interleukina-6
IL-10	Interleukin-10 / Interleukina-10
iNOS	Inducible Nitric Oxide Synthase /
	Oxido nitriko sintasaren isoforma induzikorra
IP3	Inositol Tri Phosphate / Inositol trifosfatoa
ISS	Integrated Side Scatter
K^+	Potassium ion / Potasio ioia
K+-ATPase	Potassium ATPase / Potasio ATPasa
LPA- 4	Lysophosphatidic acid receptor 4
MBP	Myelin Basic Protein / Mielinaren proteina basikoa
mRNA	Messenger Ribonucleic Acid /
	Azido erribonukleikoaren mezularia
MRI	Magnetic Resonance Imaging /
	Erresonantzia magnetiko bidezko irudigintza
N_2	Nitrogen ion / Nitrogeno ioia
Na^+	Sodium ion / Sodio ioia
Na^+/K^+	Sodium/potassium channel /Sodio/potasio kanala
NADH	Nicotinamide Adenosine Dinucleotide
NAO	10-N-nonyl-acridin orange
NMDA	N-Metyl-D-aspartate
nNOS	Neuronal Nitric Oxide Synthase / Oxido nitriko sintasa neuronala
NO	Oxide nitric / Oxido nitrikoa
NOS	Oxide Nitric Synthase / Oxido nitriko sintasa
O_2-.	Superoxide anion / Anioi superoxidoa

O_2	Oxygen / Oxigenoa
HO.	Hydroxide anion /Anioi hidroxidoa
ONOO-	Peroxinitrite / Peroxinitritoa
PB	Phosphate Buffer / Fosfato tanpoia
PBS	Phosphate Buffered Saline / Gatz fosfato tanpoia
PCR	Polymerase chain reaction /Polimerasaren kate-erreakzioa
pH	Potential of hydrogen
Rh 123	Rhodamine 123
ROS	Reactive Oxygen Species / Oxigenoaren espezie erreaktiboak
RNS	Reactive Nitrogen Species / Nitrogenoaren espezie erreaktiboak
RVT	Resveratrol / Erresberatrola
RT	RetroTranscription / Alderantzizko transkripzioa
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction / Denbora errealeko polimerasaren kate-erreakzio kuantitatiboa
SEM	Standar Error of the Mean / Batezbestekoaren errore estandarra
SOD	Superoxide dismutase /Superoxido dismutasa
SOD 1	Superoxide dismutase 1 / CuZn-superoxido dismutasa
SOD 2	Superoxide dismutase 2 / Ssuperoxido dismutasa mitokondriala
TNF	Tumor Necrosis Factor / Tumoreen nekrosi faktorea
VEGF	Vascular Endothelial Growth Factor / Endotelio baskularraren hazkuntza faktorea
ZO	Zonula Ocludens

1. SARRERA

1.1. LESIO HIPOXIKO-ISKEMIKOA: KONTZEPTUA

Entzefalopatia hipoxiko-iskemikoa (*Hypoxic-Ischemic Encephalopathy*, HIE) oxigeno eta odolaren fluxuaren etenak eragiten duen burmuineko kaltea da. Gaur egun erikortasun eta hilkortasun neurologiko perinatalaren kausa nagusienetariko bat izaten jarraitzen du, bai jaioberri goiztiarretan zein epean jaiotakoetan (du Plessis eta Volpe, 2002; Azra eta Bhutta, 2006; Jiang eta lank., 2014; Massaro eta lank., 2015).

Orokorrean garuneko lesioak asfixian du oinarria, gasen elkartrukeen arazoetan, alegia. Asfixiaren eraginez odolean oxigeno (O_2) eskasia eta karbono dioxido (CO_2) gehiegi egoten da, horrek azidosia dakarrelarik. Hala ere, burmuin kalte honen kausa etipatogenikoa zehazki zein den ezartzea zaila izaten da, ez baitago argi odoleko O_2ren murrizketa (hipoxemia deiturikoa) edo odolaren fluxuaren gutxipena (edo iskemia) den eragile nagusia (Volpe, 1995). Ziurrenik, kasu gehienetan bien arteko eraginagatik agertzen denez, praktika klinikoan lesio edo entzefalopatia hipoxiko-iskemikoa terminoa erabiltzen hasi zen.

Burmuineko oxigeno eta glukosaren murrizketaren ondorioz, jaioberria hil daiteke edo behin betiko gaitz neurologiko larriak paira ditzake, garun-paralisia, epilepsia, adimen-atzeratasuna, ikasteko zailtasunak, hiperaktibitatea eta arreta-defizita, edo ikusteko eta entzuteko gabeziak kasu (Volpe, 2001; Low eta lank., 2004; Vannucci eta Hagberg, 2004; Aridas eta lank., 2014).

1.2. LESIO HIPOXIKO-ISKEMIKOAREN EPIDEMIOLOGIA

Entzefalopatia hipoxiko-iskemikoa mundu mailako heriotz perinatalen laurdenarekin erlazionaturik dago (Tagin eta lank., 2015). 2010.urtean entzefalopatia neonatalen 1,15 milioi kasu neurtu ziren: %95 errenta baxuko edo ertaineko herrialdetan (Lee eta lank., 2013), aitzitik, garatutako herrialdetan 1000 jaioberritatik 2-6 umeek HIE pairatzen dute (De Haan eta lank., 2006; Schendel eta lank., 2012; Xiao eta lank., 2015; Mann eta lank., 2015). Argi dagoenez garapen bidean daudenetan intzidentzia askoz altuagoa da (Perlman, 2006).

Erditze goiztiarren eta haurdunaldi anizkoitzen emendioak, arrisku altuko umeen biziraupenarekin batera, lesio hipoxiko-iskemikoaren prebalentzia mundu-mailan areagotzen lagundu dute. Aurrerakuntza pedriatikoei esker heriotza-tasa asko murriztu den arren (%50-75etik %25-50ra pasa da gaur egun) biziraupenaren emendioak gaitz neurologikoak pairatzen dituzten ume-kopurua ugaritzea ekarri du (Gill eta Perez-Polo, 2008). HIEra bizirauten duten umeen %80ak arazo larriak jasaten dituzten bitartean, %10-20ak tarteko konplikazioak izaten dituzte eta soilik %10 baino gutxiago daude normaltasunaren parametrotan (Tataranno eta lank., 2015).

Aipatu bezala, hipoxia-iskemia perinatala heriotzaren eta epe luzeko desgaitasunen kausa nagusiena da, sarritan burmuinean gertatzen den kaltea konponezina izaten delako, eta honek funtzio neuronalen galera dakar, aldi berean, defizit kognitiboak eta disfuntzio motorrak azaltzen direlarik (Du Plessis eta Volpe, 2002; Hamrick eta Ferriero, 2003; Edward eta lank., 2010; Damoradan eta lank., 2014). Zoritxarrez azkeneko berrogei urtetan asfixia larri-moderatua jasaten duten umeen kopurua ez da gutxitu, ezta garun-paralisiaren intzidentzia ere (Badawi eta lank., 2005; McKenna eta lank., 2015; Wilson, 2015). HIErekin diagnostikatuak izan diren umeen artean %13ak garun-paralisia jasaten du (MacLennan, 1999; McKenna eta lank., 2015).

Ikerketa neuropatologiko eta epidemiologikoei esker hainbat arrisku faktore identifikatu izan dira amarengan, plazentan edota zilbor-hestean eta fetuarengan; hala nola, anestesia transplazentala, sortzetiko sepsia, erditzeko trauma, amak gaixotasun kardiobaskularrak edo anemia izatea, zilbor-hesteko zirkulazioa etetea, fetuaren sortzetiko biriketako eta bihotzeko anomaliak edota anemia... Asfixia perinatala aldi prenatalean (erditzean edo aurretik) edo postnatalean suerta daiteke (McGuire, 2007; Rennie eta lank., 2008).

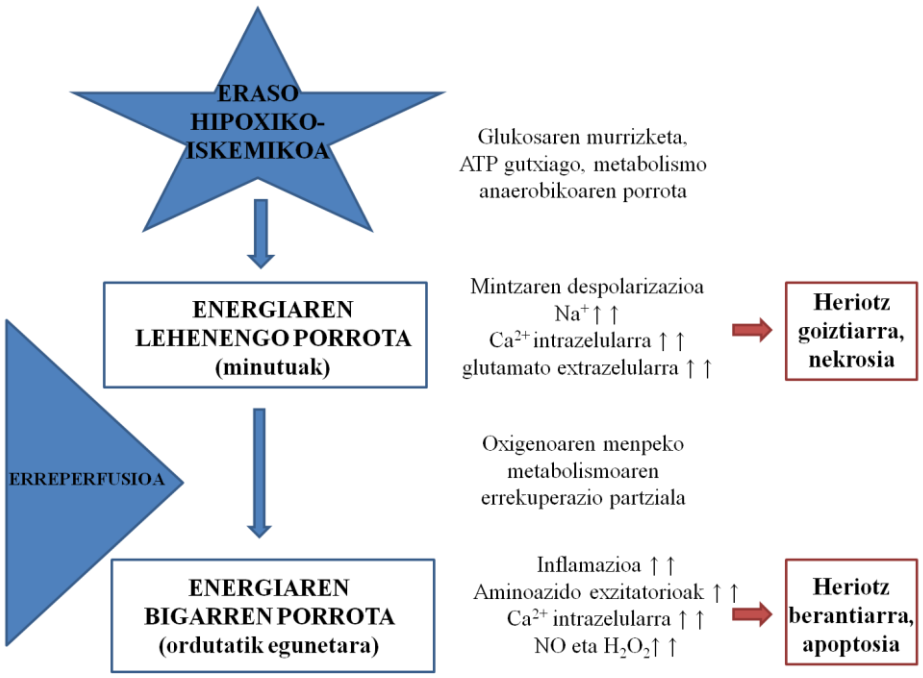
Hipoxia-iskemia neonatalaren diagnosi klinikoa bi irizpideren araberakoa da: alde batetik, jaioberriek jaio eta 5 minututara Apgar testan 7 puntu baino gutxiago erdiesten dutenean, hots, defizit neurologiko eta kardiobaskularra aurkezten dutenean, eta bestetik, odoleko pHa 7 baino baxuagoa dutenean, hau da, azidemia pairatzen dutenean. Orduan, pediatrek umeak asfixia jasan duela diagnostika dezakete, izan ere, asfixia terminoa arnas gasen trukearen desoreka eta ondorengoko azidosi metabolikoaren garapena bezala defini dezakegu (MacLennan, 1999).

1.3. KALTE HIPOXIKO-ISKEMIKOAREN PATOFISIOLOGIA BURMUIN NEONATALEAN

Kalte hipoxiko-iskemikoa gertatu aurretik, fetuaren oinarri genetikoak, egoera metabolikoak, sexuak eta haurdunaldiaren denborak (adin gestazionalak) fetuak moldatzeko dituen aukerak baldintzatzen dituzte (Jhnston eta Hagberg, 2007; Vannucci eta Hagberg, 2004).

Kalte hipoxiko-iskemikoaren kontrako lehenengo moldapen-erantzuna odol-fluxuaren birbanaketa da, biziraupenerako ezinbestekoak ez diren organoetara odoleztatzea gutxituz (hala nola, heste, giltzurrun eta biriketara) eta bihotz eta burmuina bezalako funtsezko organoetara areagotuz. Odolaren fluxuaren birbanaketa mekanismo desberdinei esker lortzen da. Alde batetik, hipoxiak eta hiperkapniak bideraturiko burmuineko hodi-zabalkuntza dago, fluxu preferentziala enbor entzefalikora bideratzen duena. Beste alde batetik, adrenalinaren mailaren emendioa ere badago, hipertentsioaren eta erresistentzia periferiko baskularraren handipena dakarrena. Azkenik, gorputzaren mugimenduak eta arnasketa gutxitu egiten dira, brakikardia ezartzen delarik mekanismo bikoitz bati esker: hipoxiaren eraginez kimiohartzaileak estimulatzen diren bitartean, hipertentsioak barohartzaileak estimulatzen ditu. Guzti honek fetuaren energiaren erabateko aurrezpena suposatzen du.

Hala eta guztiz ere, hipoxia-gertaerak larriak eta iraunkorrak direnean, konpentsazio-mekanismoak ez dira gai kalteari aurre egiteko. Burmuinaren lesioa eragiten dituzten mekanismo patogeniko nagusiak bi fasetan banatzen dira (1.go irudia). Hasierako fase bat dago, energiaren agorpenen ondorioz sortatzen dena (**hipoxia-iskemia fasea**). Gero, zenbait orduren osteko berroxigenazio eta erreperfusioak eragindako bigarren fase bat dago, turrusta biokimiko bati ekiten diona (**berroxigenazio eta erreperfusio fasea**). Bigarren fase hau egunetan zehar luza daiteke, eta batik bat, garun-azala, hipokanpoa, talamoa eta ganglio basaletako populazio neuronalak kaltetzen ditu (Azzopardi eta Edward, 2010; Aridas eta lank., 2014).



1.irudia: Eraso hipoxiko-iskemikoaren osteko faseak, bere aldaketa molekularrekin.

1.3.1. HIPOXIA-ISKEMIA FASEA

Oxigenoaren kontzentrazioa maila kritiko batera jaisten denean eta energia agortzen denean, fosforilazio oxidatiboaren bidezko ATPren sintesia inhibitu egiten da, hortaz bere aitzindariak (ADP, AMP, Pi eta H^+) metatu egiten dira. Orduan, zelulek oinarrizko funtzioak betetzeko helburuarekin metabolismo anaerobikora jotzen dute, hiru bidezidor metaboliko desberdin piztu daitezkeelarik: glukolisi anaerobioa, kreatinfosfatasaren bidea eta adenilatokinasaren erreakzioa. Baina metabolismo anaerobikoa energetikoki eraginkorra ez denez, ATParen maila murriztu egiten da, azido laktikoa eta hipoxantina bezalako metabolitoak pilatzen direlarik, eta ioi-ponpek huts egiten dute.

ATPren defizitak ioien garraio-ponpak inaktibatzen dituenean, ioien homeostasiaren galera dakar. Potasioa neuronetatik irteten den bitartean, sodioa, kaltzioa eta ura zelularen barnealdera etengabe sartu egiten dira, mintzaren despolarizazioa bultzatuz. Kloruro sodikoaren eta uraren sarrera zelularen edemarekin erlazionatzen dira, eta potasioa zelulaz kanpo metatzea, aldiz, asfixiaren osteko basodilatazioarekin, astrozitoen edema zitotoxikoarekin eta

neuronen metabolismoaren emendioarekin lotzen da. Mintzaren despolarizazioak neurotransmisore kitzikatzaileen gehiegizko askapena eragiten du, batik bat glutamatoarena, haien hartzaileak aktibatuz, eta ondorioz turrusta kitzikatzaile bat pizten da heriotza zelularra sustatuz.

Era berean, kaltzioaren sarrerak erreakzio kaltegarrien segida bat pizten du, zelularen heriotzarekin lotura zuzena daukana. Izan ere, kaltzio intrazelularraren igoerak A eta C fosfolipasak aktibatzen ditu, zelularen mintzeko fosfolipidoen gain eragiten dutenak. Horrela, mintza beraren iragazkortasuna modu itzulezin batez baldintzatuta azaltzen da. Honetaz gain, hidrolisiaren eraginez azido arakidonikoa sortzen dute. Azido hau kalte hipoxiko-iskemikoan zehar burmuinean pilatzen denez, bere kontzentrazioa kalte hipoxiko-iskemikoaren iraupenaren adierazlea da, zuzenean erlazionaturik baitaude: kalte gehien pairatzen duten burmuineko zonaldeek azido arakidonikoaren kontzentrazio altuenak aurkezten dituzte.

1.3.2. BERROXIGENAZIO ETA ERREPERFUSIO FASEA

Asfixia laburra baldin bada, berroxigenazioak zelularen metabolismoa berrezartzea ahalbidetzen du, zirkulazioa azkar berreskuratu eta hiperemia aldi bat azaltzen delarik. Bihotz-maiztasuna normalizatu, burmuineko edema 30 minututan desagertu eta azido laktikoaren ohiko balioak hurrengo egunetan berreskuratzen dira. Bestalde, hipoxia-iskemia larria bada eta 30 minutu baino gehiago irauten baditu, hainbat zelulek mintzaren funtzioa ezin dute berreskuratu, eta ondorioz zonalde horretan infartua agertzen da.

Beraz, minaren bigarren fasea, lehenengoa gertatu eta 6-48 orduren ostean azaltzen dena, berroxigenazioarekin erlazionaturik dago. Mitokondria kaltetzen da, lehenengo faseko kalteak hedatuz. Zitosoleko kaltzio askearen handipenak oxido nitrikoaren ekoizpena eragiten du eta erradikal askeak sortzen ditu. Azken hauek lipidoen, proteinen eta azido nukleikoen andeatzea eragiten dute. Energiaren agorpena, azidosia, glutamatoaren askapena, kaltzio intrazelularraren metaketa, oxido nitrikoaren (*Nitric Oxide*, NO) toxizitatea eta lipidoen peroxidazioaren konbinazioak zelulen heriotza dakar kasu askotan, bai nekrosi zein apoptosi bidez.

1.4. GARATZEN ARI DEN BURMUINAREN SENTIKORTASUNA

Burmuina gorputzeko organorik aktiboena da metabolikoki, arnasten dugun oxigenoaren %20 eta organismoko glukosaren %25 kontsumitzen ditu, gorputzeko pisuaren %2a soilik suposatzen duen arren. Arnasten dugun oxigenoaren %95a ATPra erreduzitzen da eta gainontzeko %5a oxigenoaren espezie erradikal (*Radical Oxygen Species*, ROS) bezala askatzen da. Azken hauek prozesu askoren ondorioz sortzeaz gain, hainbat prozesutan parte hartzen dute eta hortaz, kantitate txikitan funtsezkoak dira bizitzarako.

Burmuinaren garapena kontrol handia exijitzen duen prozesu konplexua da. Garatzen ari den burmuinak substratu anitz erabil ditzake energia-iturri moduan, glukosa, ketona gorputzak, gantz azidoak eta aminoazidoak kasu, zelulen banaketarako eta nukleotidoen, proteinen eta lipidoen biosintesirako. Metabolismoa erabakigarria da burmuinaren garapen eta funtzionamendurako ezinbestekoak diren prozesu zelularrak energiaz hornitzeko; hala nola, ATParen sorkuntzarako, sinaptogenesirako, neurotransmisoreen sintesi, askapen eta berreskurapenerako, ioien gradientea eta erredox oreka mantentze-lanetarako, eta mielinizaziorako (McKenna eta lank., 2015).

Garatzen ari den burmuina helduena baino sentikorragoa da kalte hipoxiko-iskemikoaren aurrean (Rice eta lank., 1981; Towfighi eta lank., 1995), bere ezaugarri bereziengatik: gantz azido asegabeen kontzentrazio altuagatik, bere oxigeno-kontsumo ikaragarriagatik, antioxidatzaile gutxitasunagatik (E bitamina eta azido askorbikoa), entzima antioxidatzaileen [katalasa, CuZn-superoxido dismutasa (*Superoxide dismutase 1*, SOD 1), superoxido dismutasa mitokondriala (*Superoxide dismutase 2*, SOD 2) eta glutation peroxidasa (*Glutathione peroxidase*, GPx)] desorekagatik, mielinizazio gutxi izategatik, ur-kantitate handiagatik, oxigenoak induzituriko basokonstrikzioagatik eta burdin erredox aktiborako duen eskuragarritasunagatik, besteak beste (Halliwell, 1992; Vannucci eta Hagberg, 2004; Sheldon eta lank., 2004; McLean eta Ferriero, 2004; Saugstad, 2005).

Laburbilduz, garatzeke dagoen burmuinak oxidazioarekiko sentikorrak diren mintzetako lipido ugari eta defentsa antioxidatzaile eskasa daukanez, erredox homeostasi ahula mantentzen du. Hala ere, baldintza fisiologikotan, jaioberrien

entzima antioxidatzaileen sistemak erredox homeostasia orekatzen du. Baldintza hipoxiko-iskemikotan, ordea, ez da horrela izaten, non sistemak huts egiten duen eta zelulak kalte honetatik babestu ezin dituen. Hortaz, faktore oxidatzaile eta antioxidatzaileen arteko oreka ahul hau apurtu egiten da, ROS eta erradikal askeak gehiegizko kopuruan daudelarik, eta azkenean, estres oxidatzailea azaltzen da.

Hipoxia-iskemiak garuna kaltetzeko dituen mekanismoak arras desberdinak dira jaioberri eta helduetan (Volpe, 1995; Edwards eta Azzopardi, 2000; Inder eta Volpe, 2000). Desberdintasun nabarienetariko bat jaioberritan burmuinaren garapen normalerako ezinbestekoak diren prozesuen eta kalte zelularra bideratzen dutenen arteko lotura da. Horrela, garapenaren eta lesio hipoxiko-iskemikoaren zenbait prozesuen artean paralelismo espaziala eta tenporala dago. Globalki garatzen ari den burmuinaren oxigeno-eskariak eta behar energetikoak helduenenak baino txikiagoak izan arren, garapen neuronal aktiboa duten zonaldeek, oxigeno asko erabiltzen dute, bereziki sinaptogenesirako. Orduan, homeostasi ionikorako eta metabolismo oxidatzailearentzako beharrezkoak diren entzimak (Na^+/K^+-ATPasa) aktibatu egiten dira. Hipermetabolismo honek hipoxia-iskemia egoeretan kontsumo energetiko oso altua suposatzen du. Garapen-prozesuan dagoen burmuina perfusioarekiko menpekoa da, ez baitauka energia eta substratuen berezko metaketarik.

Garatzen ari den burmuineko zenbait zonalde iskemiarekiko sentikorragoak dira beste batzuk baino, egoera fisiologikotan ere heltzen den odolaren fluxua txikiagoa delako haiengan. Mugako edo bukaerako baskularizazioa duten areak dira, arteria nagusien bukaerako adarrek irrigatzen dituzten zonaldeak. Gainera, asfixiarekiko sentikorragoak diren zonaldeak garapen-aldiaren arabera aldatzen dira: epean jaiotako umetan hemisferioetako garun-azaleko alde parasagitala, zehazki parieto-opzipital mailan, sentikorrena den bitartean, jaioberri goiztiarretan substatzia zuri peribentrikularra da (Pourcyrous, 1999). Azkenik aipatu beharrekoa da, umearen burmuinean berezko auto-erregulazio sistemak ez daudela erabat garaturik, denboraren poderioz garatzen doaz eta.

Entzefalopatia neonatalaren ondorioak kaltearen intentsitate, iraupena eta kokapenaren araberakoak dira (Ferriero, 2004; Juul eta Ferriero, 2014): bortizki kalteturiko umeen ehuneko txiki batek baino ez du inongo eragozpenik gabe

biziraungo, %15-20a hilko den bitartean (Levene eta lank., 1985; Goñi de Cerio eta lank., 2013).

Zelula-motari dagokionez, hipoxia-iskemiak teorikoki zelula guztiak kaltetzen dituen arren, denek ez dute modu berean erantzuten, neuronak sentikorrenak diren bitartean astrozitoak erresistenteenak dira (Northington eta lank., 2001; Hilario eta lank., 2005). Neuronek behin-behineko hipogluzemia edo hipoxia tarte arinak jasan ditzateke, baina biak batera gertatzen direnean kaltea nabariagoa bilakatzen da (Kaku eta lank.,1991). Neuronek glukogeno-metaketak ez dituztenez, astrozitoak dira beharrezko glukosa hornitzeaz arduratzen direnak. Izan ere, neuronak oso zelula selektiboak dira energia-iturriak aukeratzeko, hau da, ATPa lortzeko glukosa da onartzen duten iturri ia bakarra. Mitokondrien arnasketaren inhibizioaren ostean neuronak berehala hiltzen diren bitartean, astrozitoek glikolisi anaerobioan ekoiztutako ATPa erabiltzen dute mitokondriako mintz-potentziala mantentzeko, erresistenteagoak izaki (Bolaños eta lank., 2010). Hala ere, astrozitoak kaltetuak izaten direnean, haien biziraupena murrizteaz gain, neurotransmisore kitzikatzaileak zurgatzeko eta erradikal askeak deuseztatzeko gaitasuna mugatuta gera daiteke, eta honek, era berean, neuronen biziraupena arriskuan jartzen du (Chen eta Swanson, 2003; Alvarez-Diaz eta lank., 2007). Oligodendrozitoak ere oso sentikorrak dira, kalte hipoxiko-iskemikoaren aurrean mielinizazio prozesua eten egiten dute substantzia zurian lesioa eraginez (Rothstein eta Levison, 2005; Butt eta lank., 2014).

1.5. LESIO HIPOXIKO-ISKEMIKOAN INPLIKATURIKO ELEMENTUAK

1.5.1.ODOL-FLUXUA ETA BASKULARIZAZIOA

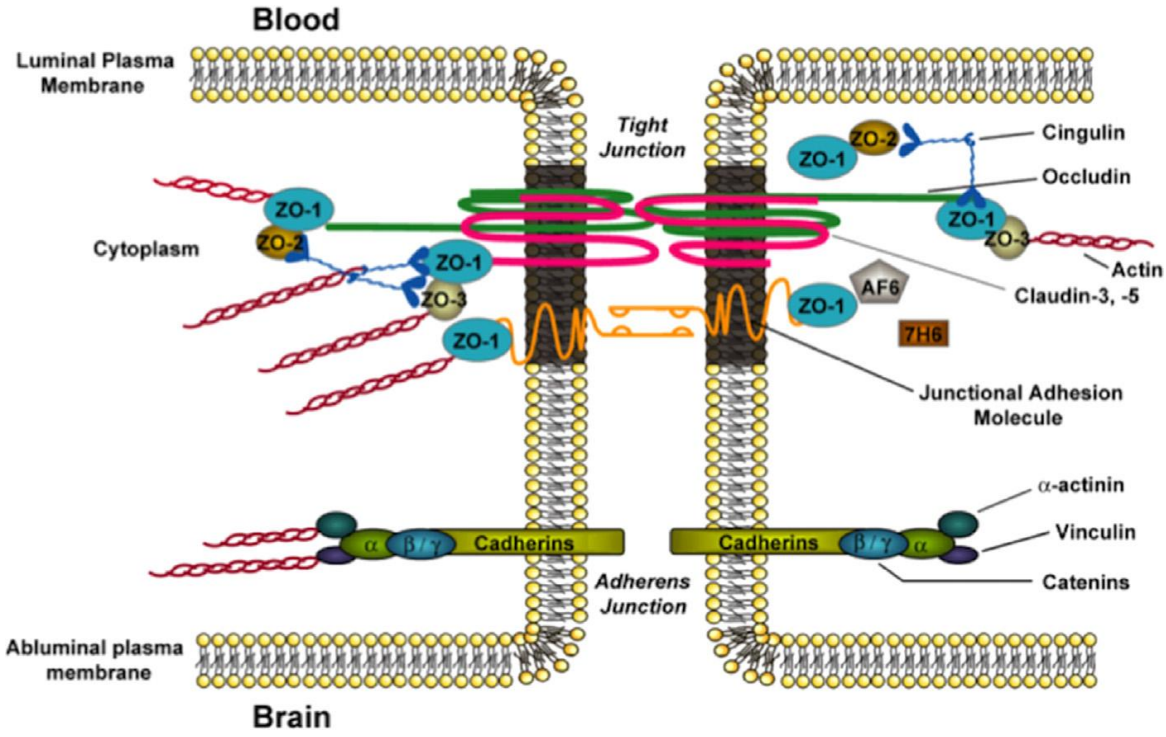
Burmuineko patologia hipoxiko-iskemikoa estuki erlazionaturik dago hipoxian zeharreko burmuineko alboko odol-fluxuaren murrizketarekin eta barrera hematoentzefalikoa (*Blood-Brain Barrier*, BBB) baldintzatuta daukaten zonaldeekin, odol-fluxua eta BBB estuki erlazionaturik daudela iradokituz (Ek eta lank., 2015).

Entzefaloak oxigeno eta glukosaren horniketa jarraitua behar du biltzeko ahalmenik ez duelako. Entzefaloa odoleztatzen duten odol-hodi gehienak bi sistema nagusiren adarrak dira, alde batetik, garuneko arteria-zirkulua, Willis poligonoa bezala ere ezagututa, eta bestetik arteria basilarra. Biak entzefaloaren oinaldean kokatuta daude, eta barneko arteria karotidatik (eskuin eta ezkerra) eta orno-arterietatik (eskuin eta ezkerra) heltzen zaie odola. Aipatu bezala burmuinak odol-fluxua birbanatzeko sistema bat du, metabolismo-aktibitate aldaketa arinen aurrean hura kontrolatzen duena. Normalean, oxigenoaren eta glukosaren urritasunen aurrean odol-fluxua enbor entzefalikora zuzentzen da, garuneko zonalde hau arduratzen baita biziraupenerako ezinbestekoak diren funtzioak mantentzeaz, arnasketa eta bihotzaren erritmoa kontrolatzen baititu.

Garapenaren fase zehatz desberdinetan zeharreko odol-hodien heltze-mailak eragin handi du lesio iskemikoak jasateko joeran. Jaioberri goiztiarrek kalte hipoxiko-iskemikoa pairatzeko predisposizio gehiago dute, aurkezten dituzten zenbait ezaugarri baskular eta fisiologikoengatik, bereziki burmuineko odol-fluxuaren erregulazio desegokia edukitzearren (Khwaja eta Volpe, 2008).

1.5. 2. BARRERA HEMATOENTZEFALIKOA

Garun kortexaren odoleztatze sistemak berezitasun garrantzitsu bat du gorputzeko gainontzeko odoleztatze sistemetatik bereizten duena, barrera hematoentzefalikoa, hain zuzen ere (2. irudia). Hipoxia-iskemiak barrera hematoentzefalikoaren molekula txiki zein handien iragazkortasuna areagotzen du erasoaren osteko orduetan, hurrengoko egunetan zehar gutxika normaltasuna berreskuratzen duelarik. Barrera hematoentzefalikoaren irekierak BBB beraren proteinen espresioaren aldaketekin loturik dago, eta era berean geneen transkripzio maila areagotzen da, kalte molekular zuzena pairatzen duela erakutsiz baina konpentsazio mekanismoak martxan ipintzen direla iradokiz. Beraz, erasoaren osteko BBBren behin-behineko irekierak patologiaren garapena sustatzen duela argi dago, baina aldi berean terapia moduan erabil daitezkeen farmakoei burmuinean infartaturiko eremuetara hobeto heltzea baimentzen die (Ek eta lank., 2015).



2. irudia: Barrera hematoentzefalikoaren adierazpen eskematikoa. Bertako bi zelula endotelialen arteko lotura-konplexua ikusten da. Ronaldson eta Davis (2012) artikulutik hartuta.

Barrera hematoentzefalikoak entzefalo-zelulak substantzia kaltegarri eta mikroorganismo patogenoetatik babesten ditu, agente horiek odoletik entzefalora pasatzea galaraziz. Nerbio sistema zentrala (NSZ; *Central Nervous System*, CNS) gainontzeko organismotik isolatzen duen muga fisikoa eta metabolikoa da, burmuinera ailegatzen diren konposatuen transferentzia zehaztasun handiz erregulatzen duena (Ronaldson eta Davis, 2015). Baldintza ez-patologikoetan lau funtzio nagusi ditu: burmuina gainontzeko organismoko konposatuetatik babestea, burmuinerako beharrezkoak diren konposatuen garraio selektiboa, odolean aldaketarik egotekotan haiek detektatzea eta garunari horien berri ematea eta garuneko eta odoleko sustantziak metabolizatzea. Laburbilduz, odoleko eta burmuineko aldaketen aurrean modu azkar batez erantzuteko gai den egitura plastikoa da (Huber eta lank., 2001).

Osagai desberdinek osatzen dute BBB, zelula endotelialek, kapilareen mintz basalek, perizitoek, astrozitoek eta mikrogliak (Ronaldson eta Davis, 2012). BBBko zelula endotelialak garuneko odol-sistema estaltzeaz gain, berezitasun bat dute, ez dira gainontzeko zelula endotelialak bezalakoak, bi desberdintasun nagusi dituztelako: ez

dute tarterik uzten zelula endotelialen artean sustantziak pasa ez daitezen eta lotura hertsiak dituzte elkarren artean. Zelula endotelialen artean ematen diren lotura hertsi hauek, NSZko endotelio baskularraren iragazkortasuna mugatzen dute plasmako solutuekiko. Horrela, urak, O_2 eta CO_2 bezalako gasek, eta zenbait molekula liposolugarri txikik baino ezin dute zeharkatu endotelio hau, garraio sistema konkretu eta arautu bat erabili gabe.

Lesio hipoxiko-iskemiko baten ostean, aminoazido neurotransmisore kitzikagarriak askatuak izaten dira BBBren oxigenoaren erradikal askeen kanalen menpeko iragazkortasunean aldaketak eraginez, sistema immuneko zelulen sarrera baimenduz eta hanturazko erantzun bat suspertuz (Moretti eta lank., 2015).

1.5.3.ASTROZITOAK

Astrozitoak NSZko glia-zelula nagusi eta ugarienak dira. Jatorri neuroektodermikoa izaki, NSZren garapenaren lehenengo garaietan sortzen dira eta garapenean zehar aitzindarien migrazioa bideratzeaz arduratzen diren zeluletan dute sorburua, glia erradialan hain zuzen ere.

Nerbio-sisteman funtzio anitz betetzen dituzte, nagusienen artean, neuronei hornidura estrukturala, trofikoa eta metabolikoa eskeintzea da; aktibitate sinaptikoan eta barrera hematoentzefalikoaren garapenean parte hartzeaz gain. Gainera, neurotransmisore kitzikatzaileen (glutamatoa) zurgapenaz, erradikal askeen deuseztapenaz, uraren garraioaz eta oxido nitriko eta zitokinen ekoizpenaz arduratzen dira (Chen eta lank., 2001; Chen eta Swanson, 2003). Laburbilduz, neuronen biziraupenerako, funtzionamendurako, neurogenesirako eta susperraldirako ezinbestekoak dira (3.irudia).

Burmuinean gertatzen diren aldaketen sentsore moduan dihardute, berehala erreakzionatzen ohi dute faktore trofikoak ekoiztuz eta ioiak zurgatuz. Hau dela eta, astrozitoen funtziotako batek huts eginez gero, neuronen biziraupena arriskuan jartzen da (Takuma eta lank., 2004). Eraso hipoxiko-iskemikoaren aurrean, astrozitoek neuronen biziraupena bermatzen dute eta astrozito-neurona arteko barne-seinalizaiorako bidezidorrek babesa eskaintzen dute. Neuronak hil eta

burmuina kaltetzean, astrozitoak ugaldu eta ehunaren errekuperazio prozesuan parte hartzen dute. Gliosi izeneko prozesu honetan, astrozitoen proliferazioak ehunean bertan eraturiko hutsuneak betetzen ditu. Gliosia ugaztun helduen NSZko birsorkuntzaren porrot nagusitzata hartu izan da. Hala ere, aldi berean astrozitoek erradikal askeak eta inflamazio bitartekariak ekoizten dituzte (Chen eta lank., 2015), zeinak potentzialki NSZko zeluletarako mesedegarriak izan daitezkeen.

Kaltearen aurrean astrozitoak aktibatuak izaten dira gliaren proteina azido fibrilarraren (*Glial Fibrilary Acid Protein*, GFAP) espresioa emendatuz (Pekny eta Nilsson, 2005). Hanturazko erantzunean parte hartzen dute, erantzun immune anti-inflamatorioak garatuz eta interleukina-2ren (IL-2) espresioa deuseztatuz; baina modu berean, inflamazioaren aldeko zeregin garrantzitsu bat ere betezen dute. Inflamazio bitartekari anitz jariatzeko gai dira, zitokinak, kimiokinak eta oxido nitriko sintasaren isoforma induzikorra (*Inducible Nitric Oxide Synthase*, iNOS) kasu (Dong eta Benveniste, 2001). Behin kalte hipoxiko-iskemikoa gaindituta, gainazaleko zenbait molekula espresatuz eta faktore trofikoak askatuz, nerbio-bukaeren hazkundea errazten dute plastizitate sinaptikoko prozesuetan, baita erregenerazio neuronalaren prozesuetan ere. Honetaz gain, astrozitoen biziraupen edo heriotzak berak nerbio-ehunaren erregenerazio-maila eta intentsitatea baldintzatzen ditu (Chen eta Swanson, 2003).

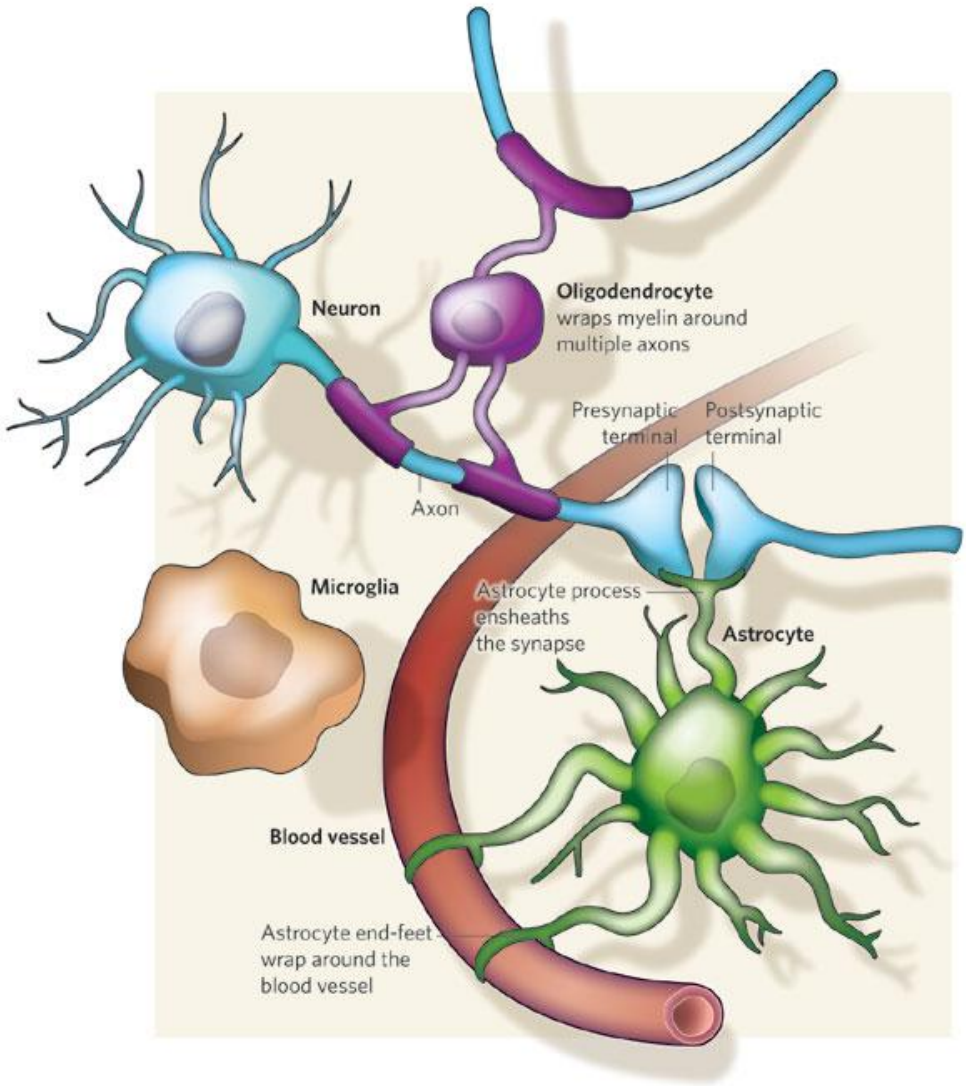
1.5.4.OLIGODENDROZITOAK

Makrogliaren parte diren zelulak dira, astrozitoak baino txikiagoak eta luzapen gutxiago dituztenak. Oligodendrozitoen funtzio nagusia axoiaren inguruko estaldura babeslea eratzea da, mielina-geruza alegia, axoien isolamendua bermatzeko (3. irudia). Mielina funtsezkoa da nerbio-bulkadak distantzia luzetara garraiatu ahal izateko. Oligodendrozitoak NSZko substantzia grisean zein zurian topatzen dira. Haien aitzindariak hodi neuralean sortzen dira eta hortik amaierako helburuetara migratzen dute, behin bertan daudelarik oligodrendozito heldutan desberdinduz, NSZko zelula mielinizatzailetan, hain zuzen ere. Oligodendrozitoen aitzindariak ugaltzeko nolabaiteko gaitasuna mantentzen dute eta zenbait seinaleen aurrean erantzun ahal izateko migratzeko gaitasun handia dute.

Garatzen ari den burmuinean, oligodendrozitoen zelula aitzindariak eta preoligodendrozitoak estres oxidatzaileari bereziki sentikorrak dira (Buonocore eta Groenendaal, 2007). Hipoxia-iskemiaren ostean, zitokinek eta erradikal askeek eragiten duten estres oxidatzailea, glutamato-hartzaileen exzitotoxizitatea, eta hanturazko erantzuna (non makrofagoak eta mikroglia ugaritzen diren) dira oligodendrozitoen kaltearen erantzule nagusiak. Honetaz gain, oligodendrozitoek estres oxidatzailea erregulatzen duten entzima antioxidatzaileen urritasuna dutela aipatu beharra dago (Ness eta lank., 2001; Noetzel eta Brunstrom, 2001).

Jaioberrietan substantzia zurian dauden axoiak mielinizatu gabe daude, hauxe bizitzaren lehenengo urtean gertatzen delarik (Gilmore eta lank., 2006). Hipoxia-iskemiak mielina kodifikatzen duten geneen espresioa kaltetzen du oligodendrozitotan (Skoff eta lank., 2001), eta substantzia zuriko axoietako asaldura honek glutamato kopuru handiak dakartza, oligodendrozitoen heriotza eraginez exzitotozitate mekanismoaren bitartez (Ness eta lank., 2001). Toxizitate hau erradikal askeen kopuru handiarekin bat ematen da, oligodendrozitoen kaltea are gehiago larrituz. Oligodendrozitoen gainazaleko zitokinentzako hartzaileen aktibazioak hauen heriotza bultza dezake. Beste alde batetik, oligodendrozitoen heltze-maila da glutamato-maila altuarekiko sentikor izateko faktorerik erabakigarriena (Back, 2014). Back eta lankideek (2002) oligodendrozitoen aitzindari berantiarrak sentikorrenak direla proposatu dute, heltzen ari direnak edo helduak baino, erradikal askeek zuzenduriko kaltean.

Eraso hipoxiko-iskemikoak oligodendrozitoen aitzindarien endekapen selektiboa eragiten du, aktibaturiko makrofago eta mikrogliaren parte hartzea dagoelarik, estres oxidatzailearekiko sentikortasunaren emendioarekin batera (Chen eta lank., 2015). Baldintza normaletan, astrozitoek eta mikrogliak oligodendrozitoen biziraupena eta desberdintzpena sustatzen dute (Nicholas eta lank., 2001), baina aktibazio egoeratan eta estimuluaren arabera kaltea eragiten diete (Pang eta lank., 2000), potentzialki toxikoak izan daitezkeen inflamazio-bitartekariak eta erradikal askeak eta oxigenoaren espezie erreaktiboak ekoiztuz (Rezaie eta Male, 2002).



3. irudia: Glia-neurona arteko elkarrekintza. Glia mota desberdinak neurona eta gertuko odol-hodiekin elkarrekiten dute. Oligodendrozitoek mielina ekoizten dute sinapsi neuronalak azkartzeko. Astrozitoek odol-hodiek eta sinapsien arteko prozesuak hedatzen dituzte. Mikrogliak burmuina infekzio eta kaltearen aurrean babesten du.

1.5.5. MIKROGLIA

Mikoroglia monozitoen linajean jatorria duen zelula-mota bat da eta NSZren gliaren populazioaren %20 suposatzen du. Beraz, jatorri mesenkimala du eta hezur muinean eratzen da. Mikroglia NSZ zehar barreiatuta dago, ganglio basaletan eta zerebeloan garun-azalean baino ugariagoa delarik. Mikroglia NSZko fagozito parenkimal mononuklearrak dira, sarritan burmuineko zelula

immunologikoak bezala ezagunak (3. irudia). Feto-garaian, NSZ gutxika populatzen doaz, bereziki 2. hiruhilabetean zehar, gero desberdindu eta adarkatu egiten delarik.

Baldintza fisiologiko arruntetan, mikroglia egoera kieszentean mantentzen da, aktibitate fagozitikorik gabe alegia, eta morfologia adarkatua azaltzen du. Bere funtzioa fluido extrazelularretan dauden neurotransmisoreak desaktibatzea da homeostasia mantentze aldera (Ronaldson eta Davis, 2012). Gaixotasun edo trauma baten aurrean, aldiz, mikroglia aktibatu egiten da garuneko lesioaren mota eta larritasunarekin arabera (Ronaldson eta Davis, 2012). Aktibazioaren ostean, mikrogliak pixkanakako aldaketa morfologikoak jasaten ditu, makrofagoen antzeko ezaugarri morfologiko eta funtzionalak bereganatzen dituelarik.

Kalte hipoxiko-iskemikoaren ostean, astrozitoek ATP askatzen dute mikroglia aktibatuz (Davalos eta lank., 2005; Nimmmerjahn eta lank., 2005). Mikrogliak odoletik etorritako makrofagoek baino erantzun azkarragoa eta eraginkorroa ematen du (Umekawa eta lank., 2015). Askotan mikrogliak astrozito eta oligodendrozitoen gaineko kaltea areagotzen du inflamazio-bitartekariak ekoizterakoan (Tahraoui eta lank., 2001).

Zitokina pro-inflamatorioak (IL-1a, IL-1b eta IL-18 esaterako) espresatzen ditu (Hagberg eta lank., 1996; Hedtjärn eta lank., 2002) eta hauek sustantzia zurian eragin negatiboak dituzte. Horien artean aipagarriak dira oligodendrozitoen aurrekarien garapenaren inhibizioa, oligodendrogliaren apoptosia eta mielinaren andeatzea. Gainera oxigenoaren erradikal askeak eta metaloproteasa-9 ekoiztu egten ditu. Honek guztiak leukozitoak burmuinean infiltratzea dakar, BBBren iragazkortasuna baldintzatuta geratu daitekeelarik (Umekawa eta lank., 2015).

Dena den, bere funtzioa hondar zelularrak deuseztatu eta fagozitatu egiten dituzte. Gainera, zelula glialen aktibazioak zenbait faktoreen sorrera dakar, tumoreen nekrosi faktore betarena (*Tumor Necrosis Factor Beta*, TNF-beta) adibidez, neuronen biziraupenerako garrantzitsua dena, eta hortaz lesioaren erregenerazioan ere parte hartzen dute. Hau guztia dela eta, oraindik ez dago guztiz argi mikrogliak efektu onuragarriak ala kaltegarriak ekartzen dituen lesio hipoxiko-iskemikoaren ostean.

1.6. BURMUIN-KALTE HIPOXIKO-ISKEMIKOAREN OINARRI MOLEKULARRAK

Hipoxia-iskemiaren ostean, gertaera metabolikoen turrusta bat sortzen da, heriotza zelularra ekar dezakeena eta askotan lesioa burmuin osora hedatzen duena (Hilario eta lank., 2006). Aipatu bezala, prozesua oxigenoaren eta odol-fluxuaren murrizketarekin hasten da, zeinak ATParen maila gutxitzen duten. Hortik aurrera, oxigenoaren espezie erreaktiboen ekoizpena, exzitotoxizitatea, kaltzio zitosolikoaren kontzentrazioaren emendioa, inflamazio-turrusta, mitokondrioaren funtzionamenduan asaldurak, heriotz zelularra mota-desberdinen bidezidorran aktibazio geniko eta transkripzionala, DNAren apurketa eta azkenik heriotz zelularra gertatzen dira (Alonso-Alconada eta lank., 2012) (4. eta 6. irudiak).

4.irudia: Kalte hipoxiko-iskemikoak eragiten dituen gertakari nagusien laburpena.

1.6.1. ENERGIAREN AGORPENA

Oxigenoaren eta glukosaren faltak fosforilazio oxidatiboa ekiditen du, eta horrek ATP erreserben berehalako agorpena eta gehiago ekoizteko ezgaitasuna dakar. ATParen deplezioaren ondoren, zelulak ez dira gai bizitzarako ezinbestekoak diren zenbait funtzio betetzeko (Hilario eta lank., 2006). Ondorioz bigarren aldaketa zelular batzuk azaltzen dira, hala nola, mintz plasmatikoaren ioi-ponpen disfuntzioa, glikolisi anaerobioaren emendioa, azido laktikoaren metaketa, kaltzio zitosoliko askearen areagotzea, ubikitinazioan urritasuna eta xantinaren metaketa.

Homeostasi ioniko eta mintz plasmatikoaren potentzialaren mantenurako Na^+/K^+ ponpen energia hornidura ezinbestekoa da, ekoizten den ATParen erdia gutxi gora-behera helburu honetarako erabiltzen baita. Mintz plasmatikoaren ioi-ponpen disfuntzioak homeostasi osmotikoaren galera dakar, Na^+, Ca^{2+} eta ura metatu egiten dira zelularen barnealdean, zelula hanpatu egiten delarik. Aldaketa hauek itzulgarriak izan daitezkeen arren, lesioa luzaroan mantenduz gero, mintza kaltetu egiten da, zelularen osotasunaren galera eta mitokondriaren disfuntzioa eraginez. Mitokondriaren endekapenak nekrosi zelularra eragiten du neuronaren funtzionaltasunaren arduraduna baita.

1.6.2. EXZITOTOXIZITATEA

Exzitotoxizitate terminoa neurotransmisore kitzikatzaileen hartzaileen gehiegizko estimulazioaren ondoriozko neuronen eta beste zelula-moten heriotza azaltzeko erabili ohi da. Neurotransmisore kitzikatzaileen gehiegizko askapenagatik, batik bat glutamato eta aspartatoarena, edota hauen birzurgatzea eraginkorra ez delako azaltzen da exzitotoxizitatea. Neurotransmisore kitzikatzaile hauek tarte extrazelularrean metatzean neurotoxina moduan jarduten baitute (Papazisis eta lank., 2008).

Azkeneko bi hamarkadetan, entzefalopatia hipoxiko-iskemikoak eragindako neuronen heriotzan glutamatoak bitartekari moduan duen garrantzia azpimarratu izan da (Walsh eta lank., 2012; Tang eta Xing, 2013). Izan ere, prozesu iskemikoak dira exzitotoxizitatea eragiten duten kausa nagusienetariko bat (Conolly eta lank., 2014).

Eragiten duten mintzaren despolarizazioak bukaera axoniko presinaptikoatik aminoazidoen askapen neurrigabea ekartzen du.

Neurotransmisoreen hartzaileak bi motatakoak dira, ianotropikoak eta metabotropikoak. Ianotropikoetan, hala nola AMPA edo NMDA, hartzaileak eta kanalak egitura bera osatzen dute, hortaz neurotransmisorea hartzaileari lotzean kanala aktibatu egiten da, neurotransmisorearen arabera erantzuna desberdina izango delarik. Metabotropikoetan, G edo ACPD kasu, hartzailea G proteina batekin loturik dago eta bigarren mezularien bidez seinalea transmititzen da. Egoera fisiologikoetan aminoazidoak deuseztatuak izaten ohi dira bukaera sinaptikoan birzurgapen mekanismo bati esker, gehienetan (%80ren kasuetan) neuronek burutzen dute birzurgapena, eta gainontzekoetan zelula glialek.

Exzitotoxizitatea hartzaileen bidez burutuko da bi mekanismoren bitartez, bata azkarra eta akutua eta bestea atzeratua (Lynch eta Guttmann, 2002). Exzitotoxizitate akutua agonista glutaminergikoek induzitzen dute eta zelularen barrualdera Na^+, Cl^- eta uraren sarrera masiboa suposatzen du, zitolisi osmotikoa eta nekrosi bidezko heriotza eraginez. Atzeratua, aldiz, kaltzio zitosolikoaren emendioak bideratzen du eta ATParen deplezioarekin erlazionaturik dago. Aurretik aipatu bezala, kaltzio zitosolikoaren igoerak entzima zitosoliko ugari aktibatzen ditu, adibidez, A2 eta C fosfolipasak, proteasak, endonukleaak eta oxido nitriko sintasa (*Nitric Oxide Synthase*, NOS), kalte zelularra eta apoptosi bidezko heriotza ekarriko dituzten hainbat turrusta metaboliko pizten dituzte (Tang eta Xing, 2013).

1.6.3. ESTRES OXIDATZAILEA

Estres oxidatzailea oxigenoaren espezie erreaktiboen ekoizpenaren eta organismoak espezie hauek modu eraginkor eta azkar baten deuseztatzeko duen gaitasunaren arteko desoreka bezala definitzen da. Estres oxidatzailea hipoxia-iskemiaren osteko burmuin-kaltearen eragile nagusitzat hartzen da (Wagner eta lank., 2004; Liu eta lank., 2015), neurotransmisore kitzikatzaile gehiegi egoteak sortutako toxizitatearen ondorioa baita.

Oxigenoaren espezie erreaktiboak oxigenoaren metabolismoko produktuak dira eta bere kontzentrazioaren arabera onuragarriak edo kaltegarriak izan daitezke gure organismorako. Egoera fisiologikotan, nerbio sistema zentral zein periferikoan barne-seinalizazio bidezidorretan eta erregulazioan erredox mezulari bezala jarduten dute. Bereziki astrozitoek eta mikrogliak sortzen dituzte kontzentrazio txikitan, eta nerona eta gliaren arteko transmisio sinaptikoa eta ez-sinaptikoa modulatzeko gai dira. Oxigenoaren espezie erreaktiboak erradikal askeek, hala nola, anioi superoxidoa (O_2-.) eta hidroxiloa (HO.), eta ez-erradikal baina toxikoek, oxigeno singletea eta hidroxido peroxido (H_2O_2), kasu, osatzen dute (Buonocore eta Groenendaal, 2007). Anioi superoxidoa, hidrogeno peroxidoa eta oxido nitrikoa hipokanpoko neuronen eta gliaren arteko komunikazioan parte hartzen dute (Allen eta Bayraktutan, 2009).

Bestalde, aipatu bezala, oxidazioetan erradikal askeak sor daitezke, elektroi-bikoterik gabeko elektroi bat duen atomo, molekula edo ioiak direnak. Elektroi-bikote bat faltan izateak ezegonkorra izatea eragiten die, eta ondorioz, egonkortasunaren bila, beste molekulei elektroi bat “lapurtzen” diete. Honek aldi berean, “lapurtua” izan den molekula ezengokortzen du, erradikal aske bihurtuz eta hauek, era berean, kaltegarri bilaka daitezkeen zenbait katea-erreakzioei hasiera eman diezaiokete. Euren arriskurik handiena zelularen bizitzarako ezinbestekoak diren konposatuak, esaterako azido nukleikoak eta mitokondrietako eta zeluletako mintzak, kaltetzea da, heriotza sustatuz (McLean eta Ferriero, 2004). Hau guztia dela eta, gehiegizko kantitatean kaltegarriak dira eta patologia ugaritan inplikaturik daude, zelulak kaltetu eta haien funtzioak eteten dituztelarik (Chan, 2001; Allen eta Bayraktutan, 2009).

Oxigenoaren espezie erreaktiboak hipoxia-iskemia neonatalaren osteko garun-kaltean inplikaturik daude. Jaioberriak, goiztiarrak zein epean jaiotakoak, estres oxidatzaileari bereziki sentikorrak direlako (Buonocore eta Groenendaal, 2007). Plasmako malondialdehido (lipidoen peroxidazioaren ondororioz ekoiztutako produktu egonkorra) kantitateak, eta nitrato/nitrito mailak bereziki altuak dira entzefalopatia hipoxiko-iskemikoa pairatzen duten umetan, erradikal askeen partea hartzea iradokitzen dutelarik (Kumar eta lank., 2008). Berroxigenazioan zehar, ROSen gehiegizko ekoizpenak defentsa antioxidatzailea erraz gainditzen du.

Estres oxidatzaileak mintzetako lipidoen oxidazioa (mintzaren iragazkortasunaren handitu eta egitura aldatuz) eta proteinen tiol taldeekiko erreakzioa (mintzetako ponpa ionikoak hondatuz) eragiten ditu. Era beran, DNAren fragmentazioa (proteinen sintesia eragotziz eta ATP-maila murriztuz), kaspasen aktibazioa, aminoazido kitzikatzaileen birzurgapenaren inhibizioa (mitokondrietako kaltea sortuz NADPH mitokondrialaren deplezioarekin batera), kaltzio zitosoliko askearen kontzentrazioaren emendioa eta karbohidratoen despolimerizazioa eragiten du (Hilario eta lank., 2006). Kalte guzti hauen eraginez zelulen nekrosia, apoptosia edota autofagia gertatzen dira.

Odol-fluxuaren berehalako berreskurapenak oxigenoa jasotzen duen ehun-zatia handitzen du, ROSen bigarren ekoizpen bat ekarriz, hauxe da erreperfusioak eragiten duen kaltea, hain zuzen ere (Rodrigo eta lank., 2013). Burmuineko mintz plasmatikoaren gantz azido poli-asegabeak bereziki sentikorrak dira erradikal askeek induzituriko peroxidazioari (Kumar eta lank., 2008), horregatik hipoxia-iskemiak eragindako estres oxidatzaileak kaltetzen duen lehenengo organoetako bat da.

1.6.4. OXIDO NITRIKOA

ROSez gain, nitrogenoaren espezie erreaktiboak ere (*Reactive Nitrogen Species*, RNS) badaude. Hipoxia-iskemian zehar, kaltzio zitosolikoaren kontzentrazioaren igoerak oxido nitriko sintasa neuronalaren (*Neuronal Nitric Oxide Synthase*, nNOS) aktiazioa dakar oxido nitrikoaren sintesia katalizatzen duena (van den Tweel eta lank., 2005; Blomgren eta Hagberg, 2006). Oxido nitrikoa erradikal aske bat da molekula nueromodulatzaile bezala diharduena.

Argininatik zitrulina eraldaketaren ostean eratzen da, konstitutiboki NOS endotelialari (*Endothelial Nitric Oxide Synthase*, eNOS) esker zelula endotelialetan eta nNOSri esker astrozito eta neuoronetan. NOS isoforma induzikorrak (iNOS) ere NO sor dezake estres zelularraren aurrean, eta hauxe RNS sekundarioa bilakatzen denean kalte neuronalari hasiera eman diezaioke, nitrazio eta nitosilazio erreakzioak errazten baititu. Berez NO ez da erreaktiboa molekula gehienentzat eta basodilataziorako funtsezkoa da, baina toxiko bihurtzen da RNS sekundarioa bilakatzean (Halliwell, 2006).

NO erradikala superoxidoekin nahastu daiteke peroxinitritoa ($ONOO^-$) eratuz, hauxe erreakziorik toxikoetako bat izaki , espontaneoki nitrogeno dioxido eta hidroxilo (OH^-) moduan metabolizatzen delako (Pryor eta lank., 2002). Beraz, oxido nitrikoak erradikal superoxidoen toxizitatea erabat areagotu dezake (Jensen eta lank., 2003; Gonzalez eta Ferriero, 2008).

Hipoxia-iskemiak eragiten duen garuneko lesioan oxido nitrikoak molekula neurotoxiko ala neurobasbesle moduan joka dezake, eta hauxe zenbait faktoreen araberakoa da: zein zelula-motak ekoizten duen eta burmuinaren zein garapen-aldian. Gehiegizko NOren ekoizpena neurotoxikoa izan daiteke, exzitotoxizitatea, hantura eta apoptosia bezalako erreakzioen turrustei hasiera emanez eta burmuineko kalte primarioa larrituz. Bestealde, zelula endotelialetako NOSek ekoiztean funtzio neurobabeslea erakusten du, burmuineko odol-fluxua mantendu, neuronen kaltea eragotzi, eta leukozito eta plaketen adhesioa saiheztuz (Liu eta lank., 2015).

1.6.5. KALTZIOAREN HOMEOSTASIAREN GALERA

Kaltzioak funtsezko papera du burmuinaren lesio hipoxiko-iskemikoaren garapenean. Kaltzio zitosolikoaren igoeraren efektu negatiboen artean nNos entzimaren aktibazioa (eta horrek dakarren oxido nitrikoaren eraketa) eta erradikal askeen sorrera daude. Aipatu bezala, honek lipido, proteina eta DNAren degradazioa dakar (fosfolipasen, proteasen eta nukleasen aktibazioagatik). Era berean, mitokondriaren kaltea areagotzen du (Douglas-Escobar eta Weiss, 2012; Sameshima eta Ikenoue, 2013; Tang eta Xing, 2013).

Hipoxia-iskemiaren ostean NMDA eta AMPA hartzaileak aktibatzen dira, aminoazido kitzikatzaileen askapenaren aurrean, Ca^{2+} zitosolikoaren kontzentrazioaren emendioa dakartzatelarik (Thorton eta Hagberg, 2015). Horrela, glutamato NMDA hartzaileekin batzean, ez dira soilik kaltzioaren kanalak irekitzen, baizik eta aldi berean mintzetako fosfolipasa A_2 eta C aktibatzen dira, eta hauek fosfoinositol bifosfatoa fosfoinositol trifosfato (IP3) eta diazilglicerol bilakatzen dute. Era berean, IP3ak erretikulu endoplasmatikoko kaltzio-kanalak zabaltzen ditu, eta kaltzioaren handipen honek kaltzio-gordailuen kanal berrien irekiera bultzatzen du, azkenean kaltzioa zitosolera masiboki ateratzen da. Modu honetan atzera-elikapen zirkulu bat sortzen da

non Ca^{2+} ATP-asa bonba erabat ez-eraginkorra bilakatzen den (Paschen eta Doutheil, 1999).

Mitokondria behar bezala ez funtzionatzeak kalte zelularrean berebiziko garrantzia dauka, mitokondria ATPren sintesirako funtsezkoa delako, superoxidoen detoxifikazio prozesuan parte hartzen duelako eta kaltzioa denbora batez bahitzeko gaitasuna duelako (Larsen eta lank., 2008). Baldintza ez-patologikotan, mitokondriatik kaltzioa zitosolera askatzen den bitartean Na^+ barneratu egiten da zitoplasmatik mitokondriara, jarraian zitoplasmara bueltatzen delarik H^+ren barneraketa ahalbidetuz. Modu honetan H^+-gradiente bat sortzen da, ezinbestekoa dena fosforilazio oxidatiborako. Funtzio hauek zuzenean erlazionaturik daude barne-mintzaren potentzialarekin (Nicholls eta Budd, 2000). Hortaz, mitokondriaren potentzialaren galerak energiaren ekoizpenaz arduratzen diren mekanismoen murrizpen bat eragiteaz gain, zelularen homeostasiaren galera dakar.

Hipoxia-iskemia neonatalari buruzko ikerketetan neuronen egituran aldaketak nabarmendu dira kaltzioaren metaketa eta lokalizazioan (Puka-Sundvall eta lank., 2000). Gainera apoptosian elementu gako diren kaspasa-3, C zitokromoa eta Bcl-2 familiako proteinak aktibatuak izaten dira hipoxia-iskemiaren ondoren (Larsen eta lank., 2008; Northington eta lank., 2001).

1.6.6. HANTURA-BITARTEKARIAK

Inflamazioak edo hanturak garatzeke dagoen burmuinaren garapen normalean eta patologikoan parte hartzen du (Hagberg eta lank., 2015). Hipoxia-iskemiak inflamazio-erantzun bati hasiera eman dakioki burmin-kaltea eraginez (Aly eta lank., 2006: Park eta lank., 2015). Zitozinek, kimiozinek eta askaturiko beste zenbait molekulek burmuinean turrustu inflamatorioari ekin diezaiokete, hilabetetan edo urtetan zehar luza daitekeen bigarren mailako neuroendekapen bat eraginez, eta defizit neuronala ekarriz (Moretti eta lank., 2015). Hau da, entzefalopatia hipoxiko-iskemikoaren garapen progresiboan parte hartuz (Palmer, 1995).

Inflamazio-zitokinek efektu toxiko zuzena edo ez-zuzena izan dezakete. Modu zuzenean oxido nitriko sintasa induzikorra, ziklooxigenasa (*Cyclooxygenase*, COX 2)

eta erradikal askeen ekoizpena handituz, eta zeharka, zelula glialak pizteko gai diren faktore neurotoxikoak ekoiztuz. Hala ere, esan bezala hipoxia-iskemiaren osteko burmuin-kaltea areagotzeaz arduratzen diren zitokina horiek ere efektu neurotrofiko onuragarriak eduki ditzakete (Liu eta lank., 2015).

1.6.7. GENEEN ETA TRANSKRIPZIO-FAKTOREEN AKTIBAZIOA

Hipoxia-iskemiak zenbait gene garrantzitsuen aktibazioa eragiten du, transkripzio-faktore espezifikoen bitartez erregulatzen dena, hala nola, hipoxiak induzitzen duen 1.go faktorea (*Hypoxia-inducible factor 1, Hif1*) (Zhu eta lank., 2014). *Hif1* bi azpiunitatez osaturik dago, Hif1α eta *Hif1β. Hif1β* konstitutiboki espresatzen da; alfa azpiunitatea giro normoxikoetan (hots, oxigenoaren presentzian) degradatu egiten da proteosomaren bitartez. Hipoxiak azpiunitate honen degradazioa ekiditen du, eta orduan heterodimeroa osatzen da gene ugariren transkripzioa aktibatuz.

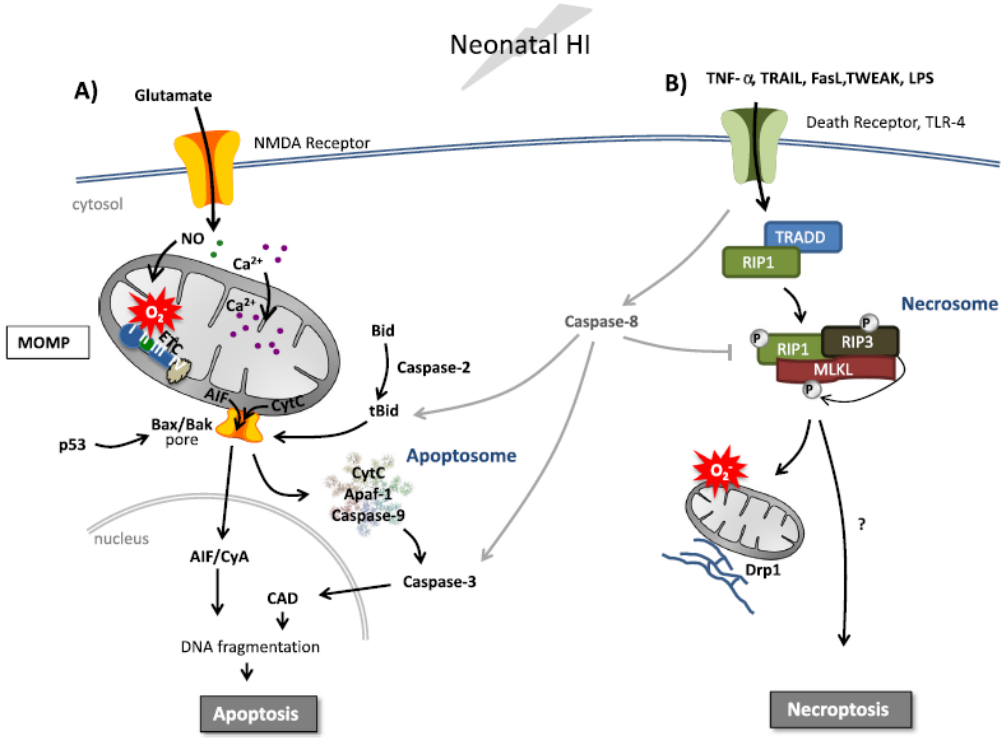
Hipoxia-iskemiaren ostean *Hif1alfak* zelulen biziraupena bermatzen du bere itu-geneak erregulatuz bai transkripzio- zein itzulpen-mailan (Li eta lank., 2014). Burmuinak iskemia pairatzen duenean, *Hif1α*-ren itu-geneak aktibatzen ditu, besteak beste, angiogenesian, glukosaren garraio eta metabolismoan eta zelulen biziraupenean parte hartzen dutenak. Endotelio baskularraren hazkuntza faktorea (*Vascular endothelial growth factor, Vegf*) *Hif1alfa*-ren itu-gene nagusienetariko bat da. Hipoxian zehar, *Hif1 α*-k berehala *Vegf* genearen transkripzioaren erregulazio-gunera batzen da eta bere transkripzio eta itzulpena pizten ditu. Sortzen den *Vegf*-k endotelioko zelulak aktibatzen ditu eta odol-hodi berriak eratzea eragiten du, ondorioz hipoxia pairatu duten burmuinaren zonaldeek oxigeno eta odol gehiago jasotzen dute (Zhu eta lank., 2014). Bestalde, eraso hipoxiko-iskemikoak mikrogliaren *Hif1α*-ren espresioa areagotu dezake eta honek mikrogliaren aktibazioa eragin dezake, aipatu bezala eragin neurotoxikoa eta neurobabeslea eduki dezakeena (Huang eta lank,, 2014).

Zenbait transkripzio-faktorek hipoxiak induzitzen dituen zenbait gene aktibatzen dituzte, hala nola: eritropoietina (EPO), eritropoiesia areagotzen duena; *Vegf*, angiogenesia eragiten duena, zenbait entzima gikolitiko, ATPren ekoizpena handitzen dutenak; *c-Fos*, c-Jun (Immediate Early Genes) espresio genikoa erregulatzen dutenak; eta *Bcl2* familiako geneak, heriotz zelularra kontrolatzen dutenak

1.6.8. HERIOTZ NEURONALA

Hipoxia-iskemiak eragindako efektu kaltegarri guztien ostean (energiaren agorpena, azidosia, glutamatoaren askapena, kaltzioaren metaketa intrazelularra, lipidoen peroxidazioa eta oxido nitrikoaren toxizitatea) zelulak hil egiten dira (Volpe eta lank., 2001; Grow eta Barks, 2002; Hilario eta lank., 2005). Hipoxia-iskemia neonatalaren ostean heriotz zelularraren hiru moten (nekrosia, apoptosia eta autofagia) ezaugarri morfologikoak aurkitu daitezke, baita neurona beran ere (Northington eta lank., 2011).

Apoptosia eta nekrosi prozesuak ikertuagoak izan badira ere, autofagia ere garrantzitsua da hipoxia-iskemiak eragindako burmuin kaltean. Hala ere, neuronen autofagiaren funtzioa zein den ez dago argi oraindik, erabiltzen diren metodoak (inhibitzaileen espezifikotasuna) egokiak ez direlako eta zalantzan daudelako (Descloux eta lank., 2015). Apoptosia eta nekrosia hipoxia-iskemiak eragindako burmuineko lesioan agerikoak dira (Northington eta lank., 2001, 2011; Han eta lank., 2000; Zhang eta lank., 2015). Heriotza apoptosi ala nekrosi bidezkoa izatea zenbait faktoreen menpekoa da (5.irudia).



5. irudia: Kalte hipoxiko-iskemikoaren osteko apoptosia eta nekrosia erakusten duen eskema, zeinek zenbait bide metaboliko partekatzen dituzte. Thornton eta Hagberg (2015).

Mitokondriaren eragina nagusia da hipoxia-iskemiak eragindako efektu guztiek bertan bat egiten dutelako (Throrton y Hagberg, 2015). Ziurrenik apoptosia edo nekrosia pizten dituzten mekanismoen aktibazioa estimuluen intentsitatearen mailaren araberakoa da. Horrela, eraso larriek nekrosia eragingo dute eta tartekoek apoptosia (Puka-Sundvall eta lank., 2000; Almeida eta Bolaños, 2001).

Honetaz gain, burmuinaren heltze-maila eta lesionaturiko zonaldeak gako dira kaltearen mekanismo eta heriotz zelularrerako. Nekrosia hipoxia-iskemiaren osteko lehenengo fasean azaltzen da, energiaren erreserbak agortzen direnean, hain zuzen ere, eta organulu eta zelula beraren hanpadura (edema zelularra) dira ezaugarri, jarraian mintz plasmatikoa haustura gertatzen delarik. Apoptosia, ordea, erreperfusioaren osteko prozesua izaten da, kaltzioa gakoa baita apoptosirako (Ali eta lank., 2014).

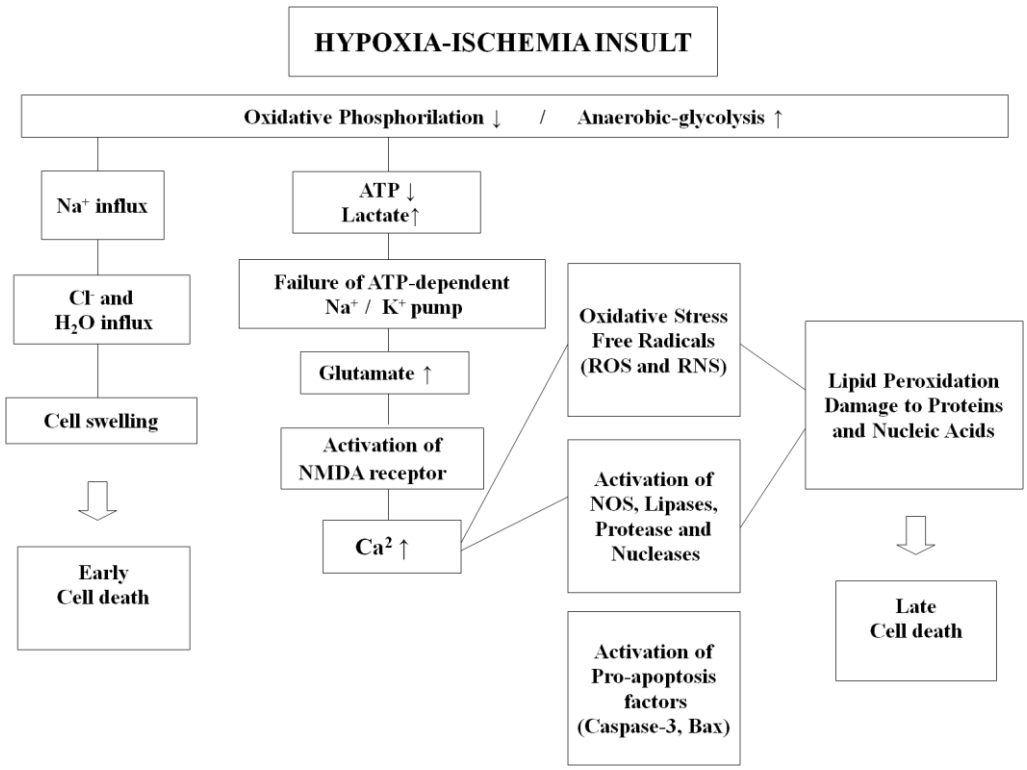
Erasoa larria denean, mitokondriaren poroaren behin betiko irekierak hanpadura eta mitokondriaren funtzioen galera ekartzen du, nekrosia berehala bideratzen da. Gainera, mitokondriaren potentzialeko asaldurek ATParen sintesia gutxitzea eta aldi berean oxigenoaren erradikal aske gehiago ekoiztea eragingo dute, nekrosi bidezko heriotza bultzatuz.

Kaltea hain larria ez denean, berriz, apoptosia gertatzen da, zeinetan mitokondriaren poroaren behin-behineko irekiera ere inplikatuta dagoen, hanpadura eta mitokondriako kanpo-mintzaren apurketa eta C zitokromoaren askapena sustatzen den, azken honek kaspasen turrusta abiaraziko duelarik (Goñi de Cerio eta lank., 2013). Geroago, poro mitokondrialaren itxierak, ATP-mailak mantentzea bermatzen du, heriotza apoptosi bidez gertatuko dela ahalbidetuz. Bcl-2 proteinen familia apopotosiaren seinale-transdukzio intrazelularrean funtsezko jarduera du, nerbio-sisteman mitokondrialen mintzeko iragazkortasuna eta poroa ere zuzenean erregulatuz. Bad, Bak, Bax, Bcl-X_s eta Bid proteinak proapoptotikoak diren heinean, Bcl-2, Bcl-X_l eta Bcl-w antiapoptotiko moduan jarduten dute, azken hauek mintzeko potentziala eutsiz eta C zitokromoaren askapena blokeatuz (Broughton eta lank., 2009; Thornton eta lank., 2012).

Mitokondria-mailan, hipoxia-iskemiak NOS entzimaren espresioa areagotzen du (Lacza eta lank., 2001) eta Bad proteina proapototikoaren desfosforilazioa induzitzen duela ikusi da, mitokondriaren kanpo-mintzatik traslokatua izaten da, poroaren irekiera

eta C zitokromoaren askapena sustatuz. Hau guztia dela eta, mitokondriak heriotza bideratzen dituzten turrustak erregulatzeko NO erabiltzen duela pentsa genezake. Eraso hipoxiko-iskemikoaren ondoren, kaspasa-3 aktibatzen arduratzen diren mekanismoa zeintzuk diren ez dago guztiz argi, mitokondria erregulatzaile nagusia dela onartuta badago ere. Kaspasen turrustari hasiera ematen dion mekanismotako bat Bad proteinaren translokazioa eta C zitokromoaren askapena dira, azken honek apoptosoman Apaf-1 (*Apoptosis protease-activating factor-1*) proteinarekin elkarri eragiten dio (Fan eta lank., 2005; Broughton eta lank., 2009; Thornton eta lank., 2012).

Nekrosi eta apoptosiaz gain, nekroptosia deiturikoa ere gertatzen da. Nekrosia ustekabeko eta kontrolik gabeko heriotz zelularra bezala definitzen da, baina nekroptosia ala programaturiko nekrosia, ordea, kontrol handiko nekrosia da (Galluzzi eta lank., 2011). Nekroptosia apoptosia induzitzen duen faktoreek (*Apoptosis Induzing Factor,* AIF) edo kaspasek bideraturiko apoptosia inhibituta dagoenean aktibatu egiten da, adibidez, birus baten erasoaren ostean edo ATParen defizita dagoenean. Sarritan TNF-alfa TRAIL edo TRL3 eta 4 bezalako heriotz-hartzaileen ligandoen atibazioaren ostean gertatzen da, bai iskemiaren ostean animalia helduetan (Vandenabeele eta lank., 2010), zein garaturik gabe dauden burmuinetan hipoxia-iskemiak jo eta gero.



6. irudia: Kalte hipoxiko-iskemikoaren laburpen-eskema adierazgarria.

1.7. HIPOXIA-ISKEMIAREN EPE LUZEKO ERAGINAK

Aurretik aipatu bezala, hipoxia-iskemiak eragindako burmuin-kalteak epe luzeko kognizio arazoak dakartza. Eragina bereziki memorian ematen da, izan ere memoriaren parte hartzen duten hipokanpoa eta kortex zerebrala dira hipoxia-iskemiaren ostean kaltetuen azaltzen diren zonaldeak (Dere eta lank., 2007; Broadbent eta lank., 2010). Hipoxia-iskemia neonatala pairatzen duten umeek adimen-atzeratasuna eta ikasteko zailtasunak izaten dituzte, haien bizitza mugatuz.

Memoria informazioa kodetu, burmuinean gorde eta handik erauzteko prozesua da. Beste era batera esanda, esperientzia bidez ikasitako informazioa gordetzeko eta informazio hori borondatez berreskuratzeko eta erabiltzeko burmuinak duen gaitasuna da.

Nerbio-sistemaren plastikotasunari esker oroimena gordetzeko gai gara. Kanpotik heltzen diren estimulu fisiko eta kimikoak zentzumenen bidez jasotzen diren arren informazioa eraldatu behar da konexio sinaptiko moduan gordetzeko eta behar dugunean erauzteko, hots, kontzientziara eramateko. Nerbio-sistema inguruneko estimuluen arabera egokitzen da erantzunak emateko, eta egokitze-prozesu hori konexio sinaptikoen kopuru eta indarren aldaketen bidez egiten da. Horri plastikotasun sinaptikoa deritzo, eta edozein oroimen gordetzeko oinarria dela uste da. Oroimena garuneko gune desberdinetan gordetzen da, gune bakoitza espezializatuta dagoelarik.

Memoria ikasketa gertatu denaren adierazle zuzena da, horregatik oroimen eta ikasketa prozesuak bereizita ikertzearen zailtasuna. Bereziki hipokanpoa (sistema linbikoaren osagaia) da bi prozesu hauetaz arduratzen den burmuineko zonaldea, bai arratoi zein gizakietan. Bi prozesu hauetaz gain, jokabide emozionalarekin eta memoria espazialarekin erlazionaturik dago.

Hipokanpoan CA 1, CA 2, CA 3 eta hortz zirkunboluzio (*Dentate gyrus*, DG) zonaldeak bereizten dira. CA 1, CA 2 eta CA3 guneek "Ammonen adarra" bezala ezagutzen den egitura osatzen dute eta bertako zelula nagusiak neurona piramidalak dira, geruzetan banatuta daudenak. Bestalde, hortz zirkunboluzioko zelula nagusiak pikor geruza osatzen duten pikor zelulak dira. Pikor zelula horien geruzak hipokanpoko zelula piramidalen geruzaren zati bat inguratzen du.

1.8. ESTRATEGIA TERAPEUTIKOAK

Azken hamarkadetan hipoxia-iskemiak eragiten dituen fenomeno biokimiko eta molekularrak hobeto ezagutzen hasi direnez, estrategia terapeutiko berriak ere garatu ahal izan dira. Orokorrean, terapia guztiek helburu berdina dute, zelulen heriotza ekiditea, organismoan inongo albo-ondoriorik izan gabe.

Entzefalopatia neonatalaren ondorioak kaltearen intentsitate, iraupena eta kokapenaren araberakoak dira (Ferriero, 2004; Juul eta Ferriero, 2014). Faktore hauetaz gain, sentikortasun iskemiko selektiboa ere badago, eta honek zelula batzuk hiltzea eta beste batzuk bizirautea baldintzatzen du, bizirauten duten zelulek penunbra iskemiko egoeran egiten dutelarik. Nahiz eta burmuineko odol-fluxua berrezartzen den, penunbra iskemikoan dauden zelula batzuk hil egingo dira, atzeraturiko heriotz zelularra dela eta (Edwards eta lank., 1997). Hala eta guztiz ere, egoera hauetan dauden zelulak izango lirateke berreskuratzeko aukera gehien dituztenak. Zelulak berreskuratu ahal izateko estrategia terapeutikoek hipoxia-iskemiak eragindako turrusta molekularren hasiera edo hedapena saihestu beharko lukete. Hori gauzatzeko leiho teraupetiko deritzon denbora tartean jardun beharko litzateke.

Gaur egun, praktika klinikoan erabiltzen den estrategia terapeutikoa hipotermia da eta burmuineko metabolismo-tasa murriztean datza. Umeen gorputzeko tenperatura modu kontrolatu batez jaitsi egiten da, 32-34ºC bitartean mantenduz. Hipotermiari esker garuneko lesioak gutxitu egiten direla frogatu egin da erresonantzia magnetiko bidezko irudigintza (*Magnetic Resonance Imaging*, MRI) erabiliz (Massaro eta lank., 2015; McNellis eta lank., 2015). Hipotermiak neuronen biziraupena emendatu, eta kaspasa-3ren eta mikrogliaren aktibazioa murrizten baititu (Garfinkle eta lank., 2015).

Hipotermiaz gain, beste zenbait farmakok eraginkortasuna erakutsi dute animaliekin egindako ikerkuntzan. Oxigenoaren erradikal askeak murrizten dituztenen artean, alopurinola, eritropoietina eta melatonina eraginkorrenak dira. Alopurinola xantina oxidasaren inhibitzailea da. Eritropoietinak propietate anti-apoptotikoak eta angiogenikoak edukitzeaz gain, hipoxia-iskemia pairatu duten arratoi neonataletan neurogenesia eragin eta efektu neurobabeslea duela frogatu izan da (Fan eta lank., 2013; Juul eta Pet, 2015). Bestalde, melatoninak burmuin-kaltea gutxitzen du eskaintzen duen neurobabesa luzaro irauten duelako (Carloni eta lank., 2008; Alonso-Alconcada eta

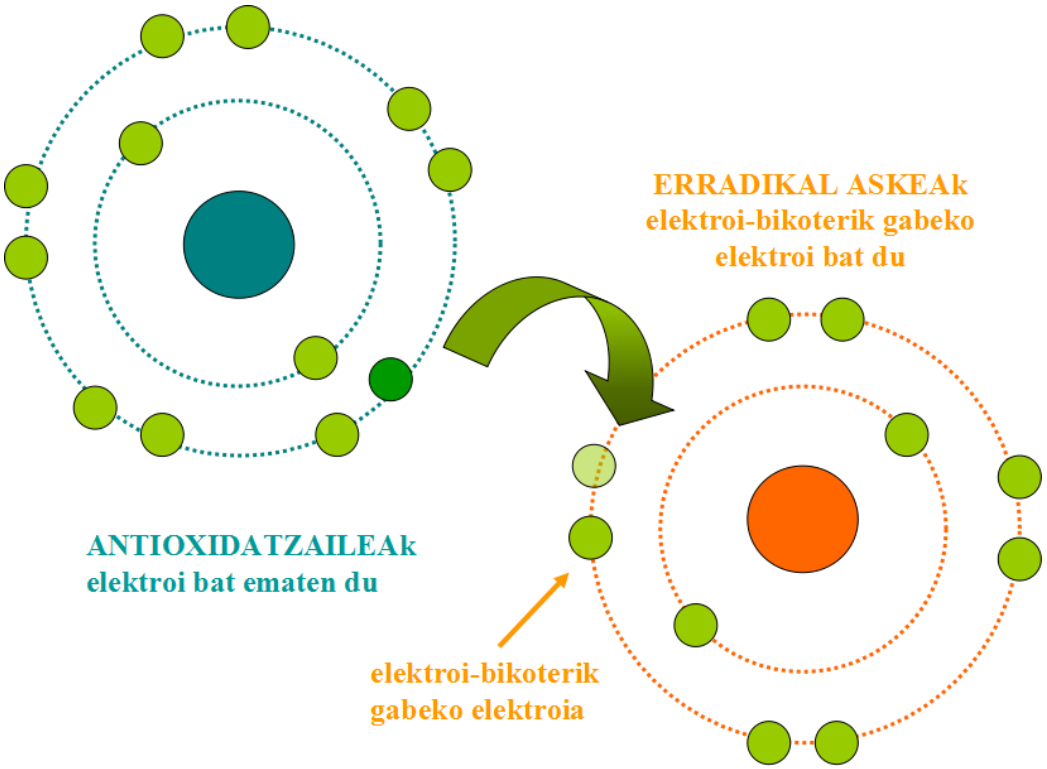
lank., 2012). Kanabinoideek ere efektu neurobabesleak dituztela erakutsi dute entzefalopatia hipoxiko-iskemikoaren animalia-eredutan (Lara-Celador eta lank., 2012). Kanabinoideen hartzaileen aktibazioak kaltzioaren kanalak itxi egiten ditu, kaltzio intrazelularraren gorakada oztopatuz (Howlett eta lank., 2004), ondorioz zuzenean hipoxia-iskemiak eragiten duen faktore baten kontra egiten dute (Volpe, 2001).

Estrategia terapeutikoak ugariak badira ere, hipoxia-iskemiaren patofisiologiaren konplexutasuna dela eta, neurobabesa modu eraginkor batez eskaintzeko etorkizunean estrategia sinergikoak erabiltzea aukera bat izan zitekeen.

1.8.1. ANTIOXIDATZAILEAK

Hipoxia-iskemiak eragindako burmuin-kaltea murrizteko erabili daitekeen terapietako bat antioxidatzaileetan oinarritzen da.

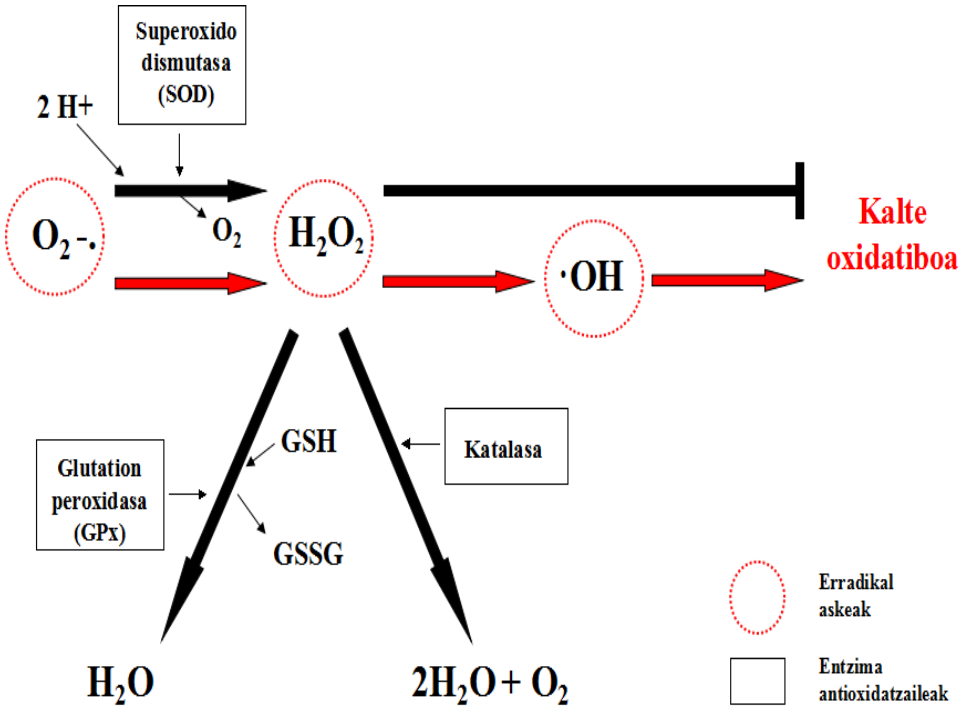
Antioxidatzaileak, bere izenak iradokitzen duen moduan, beste molekula batzuen oxidazioa atzeratu ala ekiditen duten molekulak dira. Aurretik azaldu bezala, estres oxidatiboak hipoxia-iskemiak eragindako kaltean berebiziko garrantzia du. Antioxidatzaileek erradikal askeen kopurua murrizten dute, beharrezkoak dituzten elektroiak emanez eta erreakzio kaltegarriak eragotziz (7. irudia). Antioxidatzaileen berezitasuna da elektroiak ematen dituztela euren burua oxidatuz, baina ingururako erreaktibitate gutxi edo eza duten molekula bihurtuz. Espero zitekeen bezala, antioxidatzaileen eta erradikal askeen arteko elkarrekintzak, lipido, proteina eta azido nuklekikoak bezalako substratu biologikoak kaltetuko lituzketen prozesu oxidatiboen hasiera edo hedapena prebeni dezake. Eboluzioan zehar estres oxidatiboari aurre egiteko sistema antioxidatzaileak garatu dituzte izaki bizidunek.



7. irudia: Antioxidatzaileak elektroi bat ematen dio erradikal askeari bere burua oxidatuz, honek elektroi-bikoterik gabeko elektroia bat daukalako, beste molekula baten oxidazioa ekidituz.

Gizakiak eta saguak bezalako ugaztunen kasuan sistema antioxidatzailean hainbat molekula eta entzimek parte hartzen dute.

Entzimen artean superoxido dismutasa, glutation peroxidasa eta katalasa bezalako entzima antioxidatzaileak. Bere elkarlanaren bitartez anioi superoxidoek (zenbait prozesu metabolikotan askatzen da, batez ere mitokondriako elektroien garraio-katean) eragindako kalteak ekidi daitezke. Lehenengo eta behin hidroxido peroxido bilakatzen da SOD entzimari esker. H_2O_2ren metaketa zelularentzat toxikoa izango litzatekeenez, uretara erreduzitzen da, katalasa ala GPx entzimen bitartez. Glutation peroxidasak glutationa (GSH) oxidatzen du glutation disulfuroa (GSSG) sortuz, baina hauxe bere forma erreduzitura erraz buelta daiteke beste entzima bati esker, glutation erreduktasari esker, hain zuzen ere. H_2O_2 neutralizatzen ez bada, hidroxilo bilaka daiteke, ezin dena ezabatu erreakzio entzimatiko baten bitartez, eta ondorioz, kalte oxidatiboa eragingo lukeena (8. irudia).



8.irudia: Entzima antioxidatzaileen eragina oxigenoaren espezie erradiaktiboen kontra.

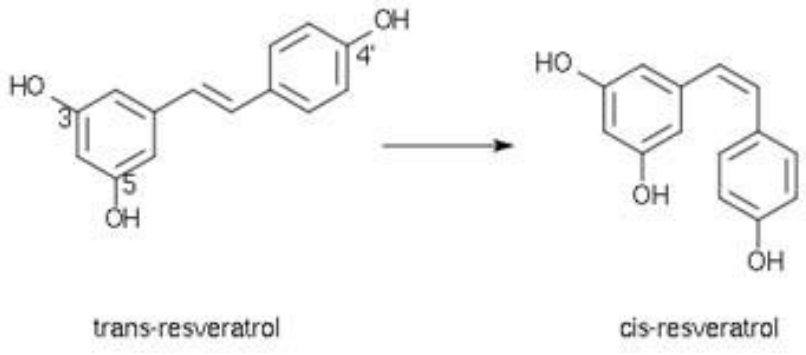
Konposatu antioxidatzaileen artean aipagarriak dira A, C, eta E bitamina, karotenoideak, azido lipoikoa, glutationa eta ubikitinak. Horietariko batzuk organismoak sintetizatzen ditu baina beste batzuk dietaren bitartez jasotzen ditugu.

Kalte oxidatiboa bizia denean, denboran zehar irauten duenenean eta ezin denean itzuli hainbat patologia eragin ditzake, hala nola kardiobaskularrak eta neuroendekapenezkoak (Alzheimer edota Parkinsonen gaitzak, esaterako). Gaur egun, antixodatzaileen erabilera zenbait gaixotasunen tratamendurako ikertzen ari da eta era berean dietatik eskuratzen ditugun antioxidatzaileen gabeziarekiko harremana. Izan ere, antioxidatzaileen onuren artean nerbio-sistemaren funtzionamendua hobetzea, ikusmen osasuntsua mantentzea, sistema immunea eta organismoko babesa indartzea, zahartzaroaren aurkako efektua izatea, gaitz kardiobaskularren eraginak gutxitzea eta urritasun kognitiboak murriztea dira ezagunenak.

Beraz, hipoxia-iskemia neonatalari aurre egiteko antioxidatzaileak estrategia terapeutiko moduan erabiltzea erabaki genuen. Horretarako dietatik soilik eskuratu ditzakegun bi antioxidatzaile aukeratu genituen: erresberatrola (RVT) eta azido dokosahexaenoiko (DHA).

1.8.1.1.ERRESBERATROLA

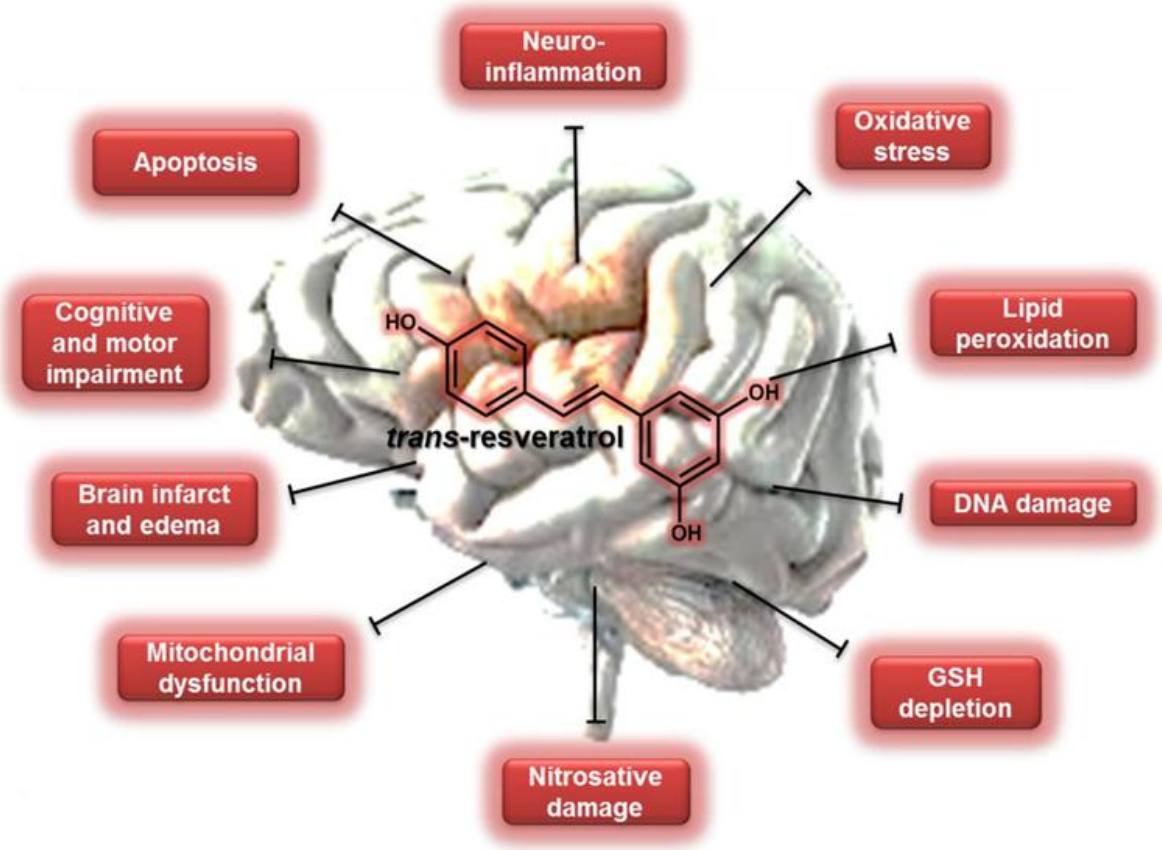
Erresberatrola konposatu polifenoliko ez flabonoidea da, metilo zubi batez loturiko bi eraztun aromatikoz osaturik dagoena (3,5,4′-trihydroxystilbene) (9.irudia). Hainbat landarek ekoiztu egiten dute patogenoen aurka edo estresari aurre egiteko babes-mekanismo moduan. Funtzio hori betetzen duten fitoalexinen familiako molekula da. Osotara 72 landare espeziek ekoiztu egiten dute mahatsondoek, pinudiek, kakahueteek, sojak eta mingranek kasu (Smoliga eta lank., 2011; Pallas eta lank., 2013).



9.irudia: Erresberatrolaren formula kimikoa. Trans eta cis isoformak agertzen dira.

Gizakiongan elikagaien bidezko erresberatrol iturri nagusia ardo beltza da. Ardo beltzak zuriak edo gorriak baino RVT kontzentrazio gehiago du. Ardo batek, hartzitze prozesuan zehar ardoa eta mahatsaren azalak elkarrekin kontaktuan zenbat eta denbora gehiago eman, orduan eta erresberatrol gehiago edukiko du. Zientzialarientzat RVT ikerketarako erakargarria bilakatzen hasi zen “paradoxa frantsesaz” deiturikoaz ohartu zirenean. Paradoxa frantsesaren arabera, Frantziako populazioak gaitz kardiobaskularren intzidentzia baxua dauka nahiz eta gantz azido ase ugari dituen dieta bat jarraitu, eta honen arrazoia ardo beltza edatea izan zitekeen, hau da, erresberatrolaren propietate kardiobabesleak (Liu eta lank., 2007; Catalgol eta lank., 2012).

Bere aktibitaterik garrantzitsuena antioxidatzailea izan arren (Nabavi eta lank., 2014), eragin anti-apoptotikoak eta anti-inflamatorioak ere baditu (Gülçin, 2010; Bastinetto eta lank., 2014; Venturini eta lank., 2010) hainbat seinalizazio-bide modulatzeko gaitasunari esker (Sinha eta lank., 2002; Yousuf eta lank., 2009; Li eta lank., 2011) (10.irudia).



10. irudia: Erresberatrolaren iskemiak eragindako kalteak apaltzeko ituak, burmuinaren infartua, edema, disfuntzio mitokondriala eta defizit kongnitiboaka eta motorrak saihesten dituen bitartean, oxigenoak eta nitrogenoak eragindako kaltea eta DNA kalteak gutxitzen ditu, neuronen apoptosia eta inflamazioa leunduz. Singh eta lank., 2013 artikulutik ateratakoa.

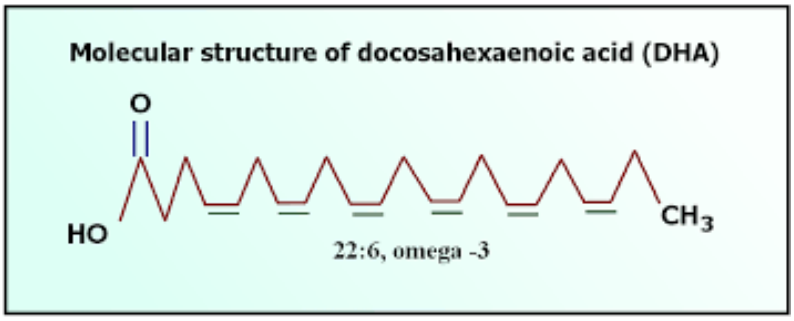
Polifenol honek neuroendepanarekin erlazionaturik dauden zenbait gaixotasunetan neurobabesa eskaintzen duela argi ikusi da behintzat maila esperimentalean, hala nola Alzheimerrean, Parkinsonean eta Huntington gaitzetan (Vingtdeux eta lank., 2008; Albani eta lank., 2010; Ferretta eta lank., 2014; Rege eta lank., 2014). Modu berean iskemian eta hezur-muin eta burmuinaren kaltean ere bere efektu neurobabesleak frogatuak izan dira (Mokni eta lank., 2007 eta 2013; Liu eta lank., 2011; Wang eta lank., 2013; Kesherwani eta lank., 2013). Efektu neurobabesleen artean aktibitate antioxidatzailea da garrantzi gehien daukana, bere estilbeno egiturari esker. Erresberatrolaren eraztun fenolikoek erradikal aske ugari deuseztatzeko gai dira, esaterako, lipido peroxilo eta erdian karbono talde bat daukaten erradikalak, eta oxigenoaren espezie erreaktiboak. Honetaz gain, zenbait entzima antioxidatzaileen

espresioa eragiteko ere gai da, hala nola SOD eta GPx entzimak (Della-Morte eta lank., 2009; Bourque eta lank., 2012; Wei eta lank., 2014). Azkeneko ikerketen arabera erresberatrolak erredox bidezidorren arduradun genikoak modulatzen ditu (Pallas eta lank., 2013).

Arratoi helduen iskemia eredutan, polifenol honek burmuinaren metabolismo energetikoa hobetuz eta estres oxidatzailea baretuz dihardu, oxigenoaren erradikal askeen eta oxido nitrikoaren ekoizpena, lipidoen peroxidazioa eta estresarekin erlazionaturiko proteinen espresioa gutxitzen dituelako (Yousuf, 2009; Simao eta lank., 2011; Wang eta lank., 2014). Hipoxia-iskemia pairatu duten arratoi jaioberrien burmuinetan, erresberatrolak bere aktibitae anti-apoptotikoa erakusten du, hipokanpoko eta garun-azaleko kaspasa-3 entzimaren aktibazioa murriztuz eta kalpainaren aktibazioa erregulatuz (Sinha eta lank., 2002; Loren eta lank., 2005; Agrawal eta lank., 2011).

1.8.1.2.AZIDO DOKOSAHEXAENOIKOA (DHA)

Azido dokosahexaenoikoa omega-3 taldeko nahitaezko gantz azido poli-asegabea da (22:6n-3) (11.irudia). Nahitaezko gantz azido bat da, derrigorrez dietaren bitartez eskuratu behar dugulako bai hura zein bere aurrekaria ere, katea motza daukan azido alfa-linoleikoa (18:3 n-3). Batik bat izokina eta atuna bezalako arrainetan eta zenbait algetan aurkitzen da. Jatorria mikroalga heterotrofiko fotosintetikotan duenez, elika-katean zehar kontzentratzen doa.



11. irudia: DHA gantz azido poli-asegabearen formula kimikoa eta egitura molekularra.

Omega-3 gantzen eskasiak burmuineko aldaketa biokimikoak eta ikusmen asaldurak sortzen ditu (Green eta Yavin, 1998; Salem eta lank., 2001; Lukiw eta Bazan, 2008; Carlson, 2009; Orr eta lank., 2013). Gizakiotan, DHA kontzentrazioa odolean urria den bitartean, burmuinean, erretinan eta espermatozoidetan oso altua da, ugaztun helduen burmuineko gantz azido poli-asegabe ugariena izateraino. Burmuineko zelulen mintzeko fosfolipidoen %30a suposatzen du, hiru fosfolipido nagusien parte izaki (fosfatidilserina, fosfatidiletanolamina eta etanolamina plasminogenoan).

Azido dokosahexaenoikoak mintz plasmatikoari jariakortasuna eskaintzen dio sinapsi gunetan, mintzaren osotasuna eta ondorioz neuronen kitzikaberatasuna eta funtzio sinaptikoa mantentzeko funtsekoa izanez. Hau guztia dela eta, DHA ezinbestekoa da mintzaren iragazkortasun ionikoaren mantenurako eta transmintzeko hartzaileen funtziorako, zeinek transmisio sinaptikoak eta gaitasun kognitiboak mantentzen dituzten (Salem eta lank., 2001; Gomez-Pinilla, 2008; Wurtman, 2008; Davis-Bruno eta Tassinari, 2011).

Garatzeke dagoen burmuinean DHAren murrizketak neurogenesian, neurotransmisoreen metabolismoan eta ikasteko eta ikusteko gaitasuna asaldurak eragiten ditu (Innis, 2008). DHA haurdunaldiko azkeneko aldian eta jaiotzaren lehenengotan metatzen da, gizakiotan haurdunaldiko hirugarren hiruhilabetetan eta arratoitan ernaldiaren azkeneko egunetan, hain zuzen ere (Suganuma eta lank., 2013). Zenbait ikerketa klinikoen arabera jaioberriak gantz azido omega-3-rekin elikatuak izateak berebiziko garrantzia du, horregatik amak haurdunaldian eta edoskitzaroan minimo batzuk mantendu behar ditu, plazenta eta esnearen bidez umeari pasatu ahal izateko. Izan ere, amaren balio horien arabera jasoko ditu umeak, eta eskuratutako DHA mailaren arabera garatuko du burmuina eta ikusmena (Innis, 2008; Guesnet eta Alessandri, 2011). DHAk efektu antioxidatzailea izateaz gain, anti-apoptotikoak eta anti-inflamatiorioak ere baditu, eta zenbait patologietan, neuroendekapenezko gaitzetan kasu, bere eraginkortasuna frogatua izan da.

2. HIPOTESIA

Entzefalopatia hipoxiko-iskemikoak, oxigeno eta glukosaren murrizketaren ondorioz azaltzen dena, erikortasun eta hilkortasun perinatalaren kausa nagusienetariko bat izaten jarraitzen du, jaiotze goiztiarrak eta haurdunaldi anitzak ugaritu diren bitartean estrategia terapeutikoen eraginkortasuna modu berean egin ez duelako.

Hipoxia-iskemia perinatalak zelulen eta ehunen kaltea ekarriko duten hainbat gertakariei hasiera ematen die. Horien artean estres oxidatzailea eragile nagusiena da, horregatik hura deuseztatzea funtsezkoa da. Garaturik gabe dagoen burmuina erabat sentikorra da kalte mota honen aurrean, gantz azido asegabeen kontzentrazio oso altua daukalako eta superoxido dismutasa eta glutation peroxidasa bezalako entzima antioxidatzaileen urritasunagatik. Honetaz gain, sistema antioxidatzailea haurdunaldiko hirugarren hiruhilabetera arte guztiz garatzen ez denez, terapia antioxidatzaile batek hipoxia-iskemiak sortutako kalte zelularra saihesten lagun zezakeen.

Gure hipotesia kalte hipoxiko-iskemikoan parte hartzen duten hasierako mekanismo zelular eta molekularren efektuak erresberatrola eta azido dokosahexaenoiko antioxidatzaileen administrazio terapeutikoari esker itzuli egin daitezkeela da. Gainera, efektu horiek erabiliko dugun arratoi-eredu esperimentalaren bitartez froga daitezkeela.

3. HELBURUAK

Lan honen helburu nagusia arratoietan hipoxia-iskemiak eragindako burmuin-kaltean erresberatrola eta azido dokosahexaenoiko antioxidatzaileen eraginkortasuna, bai indibidualki zein modu konbinatu batean, aztertzea izan zen.

Horretarako, kaltea eta antioxidatzaileen efektua baloratu egingo dugu helburu zehatz hauek jarraituz:

1. Epe ertaineko ikerketa morfologiko bat burutzea, hipoxia-iskemiak burmuinean eragindako kaltea antioxidatzaileek itzultzeko ahalmena duten frogatzeko. Horretarako kaltea kuantifikatu eta NSZko zelula-mota desberdinak aztertuko ditugu (neuronak, astrozitoak, oligodendrozitoak eta mikroglia).

2. Burmuin-kalte hipoxiko-iskemikoaren larritasuna zehaztea zenbait markatzaile biologikoen bitartez, epe laburrean hipoxia-iskemia gertatu osteko une zehatzetan (0 h, 3 h eta 12 h).

   - Mitokondrialaren barne-mintzaren osotasuna
   - Mitokondrialaren mintzaren potentziala
   - Oxigenoaren erradikal askeen ekoizpena

3. Zenbait geneen espresioa modu kuantitatibo batez neurtzea epe laburrean, hipoxia-iskemia osteko une zehatzetan (0 h, 3 h eta 12 h)

4. Kalteak eragin dezakeen portaera aldaketak aztertzea eta epe luzera antioxidatzaileen administrazioarekin hobekuntzarik ikusten diren ebaluatzea, horretarako lau portaera parametrotan bereziki arreta jarriz:

   - Aktibitate lokomotor orokorra
   - Aktibitate miatzailea, antsietatea eta neofobia
   - Landutako memoria espaziala
   - Landutako ikuste-memoria (ez-espaziala)

5. Arratoi heldutan burmuin-kalte hipoxiko-iskemikoaren osteko eta antioxidatzaileen administrazioaren osteko konexio neuronalak epe luzera ikertzea, trazatzaile aurrekarien bitartez.

4. MATERIALAK eta METODOAK

4.1. ANIMALIAK ETA ETIKA IKERKETAN

Ikerketa honetan erabilitako animaliak Sprague-Dawley arratoiak (*Ratus norvegicus*) izan ziren. Arratoiei eginiko prozedura esperimental guztiak Europar Batasuneko ikerketako eta animalien maneiurako argibideak jarraituz egin ziren (2010/63/EU). Era berean, prozedura eta protokolo esperimental guztiek Euskal Herriko Unibertsitateko (EHU/UPV) Animalien Ongizaterako Etika Batzordearen (AOEB) onarpena jaso zuten.

Animaliak unibertsitate honetako animaliategian mantendu ziren, 12 orduko argi eta iluntasun zikloekin, ura eta janaria edozein momentutan eskuragarri zutelarik, eta oinazeak, sufrimenduak, larritasunak edo lesio luzeak saihesteko eta animalia kopurua ahalik eta gehien murrizteko neurriak hartu izan zirelarik.

4.2. KALTE HIPOXICO-ISKEMIKOA: RICE-VANNUCCI EREDUA

7 egun zituztenean (P7) arratoiei hipoxia-iskemia eragin zitzaien Rice-Vannucci (Rice-Vannucci, 1981) eredua erabiliz, bi fasetan banatzen dena. Hortaz, burmuin-kaltea eragiteko, lehenengo eta behin, ezkerreko arteria karotida komuna behin betiko okluitu egin zitzaien, eta bi ordutako atseden prozesu baten ostean, animaliak 135 minutuz asfixiatu egin ziren oxigeno kontzentrazioa %8-ra jaitsiz.

Jaiotzaren ostean kumaldiak 10 arratoietara normalizatu ziren, eta zazpi egunen buruan, hipoxia-iskemia 8 animaliei baino ez zitzaien eragin. Horretarako, arratoiak isofluoranoaren bitartez anestesiatu ziren, hasieran indukziorako %3.5-tan eta gero mantenurako %1.5-tan. Ezkerreko karotida komuna behin betiko okluitzeko bi korapilo egin genizkion 6/0-ko haria erabiliz eta jarraian kauterizadore baten bitartez kauterizatu genuen. Ondoren, zauria 3/0-ko hariarekin itxi eta desinfektatu genuen klorhexidinarekin (%0,5). Arratoiek beroa galtzea ekiditeko, uneoro tapaki elektriko baten gainean jardun genuen. Ebakuntza bakoitzeko emandako denbora ez zen 8 minutukoa baino gehiagokoa izan (12.irudia).

12.irudia: Arratoiei ezkerreko arteria karotida komuna okluitzen zitzaien uneko argazkia, lupa bitartez behatzen ziren eta anestesiarako gailua ere ikus daiteke.

Behin arratoi guztiei ebakuntza egin genielarik, amarekin bueltatu ziren, bi orduz atseden hartzeko eta elikatzeko, kumeak indarberritzeko. Ostean hipoxiari ekin genion, horretarako kumeak launa 1000 ml-ko bi ontzitan sartu eta hermetikoki itxi egin genituen (13.irudia). Ontziak 36,5ºC-tara berotutako bainuan sartu ziren eta oxigenoa %8-ra murriztuta zuen airea bertatik pasarazi zen 135 minutuz. Denbora horren ostean, kumeak bere amarekin bueltatu ziren, ikerketa denbora zehatzaren unera arte.

13.irudia: Hipoxia eragiteko erabiltzen zen sistemaren argazkia.

4.3. TRATAMENDUAK

Ikerketa honetarako proposaturiko tratamenduak erresberatrola eta azido dokosahexaenoikoa antioxidatzaileak izan ziren. Iskemia eragindako animaliak atsedenean zeudenean, indibidualki pisatu eta hipoxia baino 10 minutu lehenago emango zitzaion tratamenduaren dosia kalkulatu zen. Dosi eraginkorrena bilaketa bibliografikoaren bitartez aukeratu zen.

Erresberatrola (Sigma-Aldrich, St Louis, Mo, AEB) 20 mg/kg-ko kontzentrazioan DMSO (Sigma-Aldrich) disolbatuta eta serum salinoan diluituta (West eta lank., 2007) eman zitzaien bitartean, DHA (Sigma-Aldrich) 1 mg/kg-ko kontzentrazioan giza albuminan (Sigma-Aldrich) disolbatuta (Berman eta lank., 2010) erabili genuen, eta azkenik, bi antioxidatzaile hauen konbinaketa administratu genien, bakoitzaren zegokion kontzentrazioan.

4.4. IKERKETA DENBORAK

Kalte hipoxiko-iskemikoa eta tratamenduen eraginkortasuna aztertzeko, hipoxia-iskemiaren osteko ikerketa denborak honako hauek izan ziren (1.go taula):

1.go taula: Ikerketa denborak, bere ordu eta egun zehatzekin eta erabilitako teknikak.

Ikerketa denbora	Orduak/egunak	Teknikak
Epe laburra	0 h, 3 h eta12 h (P7)	Fluxuzko zitometria
		RT-qPCR bidezko espresio genikoa
Tarteko epea	7 egun (P14)	Histologia
		Immunohistokimika eta Immunofluoreszentzia
Epe luzea	83 egun (P90)	Portaera testak
	93 egun (P100)	Trazatzaileak

4.5. IKERKETA TALDEAK

1. Kontrol taldea (Kontrola): Ikerketa talde honetako animaliek ez zuten ebakuntzarik izan, baina anestesiatu eta zauri bat egin zitzaien, gainontzeko animaliak bezala isofluoranoarekin kontaktuan egoteko eta zauri bat orbaintzeko beharra izateko.

2. Talde hipoxiko-iskemikoa (HI): Animalia hauei ezkerreko arteria karotida komuna behin betiko okluitu egin zitzaien eta 135 minutuz asfixiatu egin ziren.

3. Erresberatrolarekin trataturiko talde HI (RVT): Animalia HI-ei erresberatrola DMSO disolbatuta eta serum salinoan diluituta, 20 mg/kg-ko kontzentrazioan intraperitonealki injektatu zitzaien dosi bakar baten bidez (50 µl), hipoxia eragin baino 10 minutu lehenago.

4. Azido dokosahexaenoikoarekin trataturiko talde HI (DHA) : Arratoi HI-ei DHA giza albuminan disolbatuta 1 mg/kg-ko kontzentrazioan intraperitonealki injektatu zitzaien dosi bakar baten bidez 50 µl-ko bolumenean, hipoxia eragin baino 10 minutu lehenago.

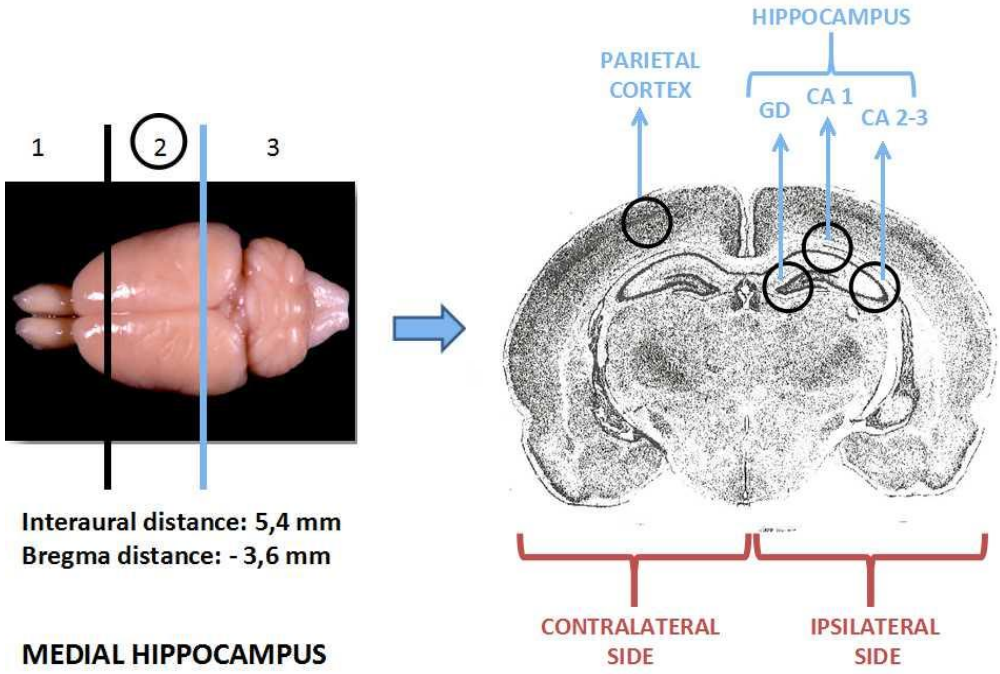
5. Resberatrol eta azido dokosahexaenoikoaren konbinazioaz trataturiko talde HI (RVT+DHA): Animalia HI-ei erresberatrola (20 mg/kg) zein DHA (1 mg/kg) intraperitonealki injektatu zitzaien dosi bakar baten bidez, hipoxia eragin baino 10 minutu lehenago.

Ikerketa desberdinetan talde bakoitzeko erabilitako animalien kopurua hurrengo hau da:

- Ikerketa morfologikoa: n=8
- Fluxuzko zitometria bidezko ikerketa: n≥5
- RT-qPCR bidezko espresio genikoaren ikerketa: n=5, hiru kopiekin
- Portaeraren azterketa: Kontrola (n=16), HI (n=14), RVT (n=10), DHA (n=14), RVT+DHA (n=9)
- Neuronen konexioak aztertzeko trazatzaile aurrerakoien (n≥3) ikerketa.

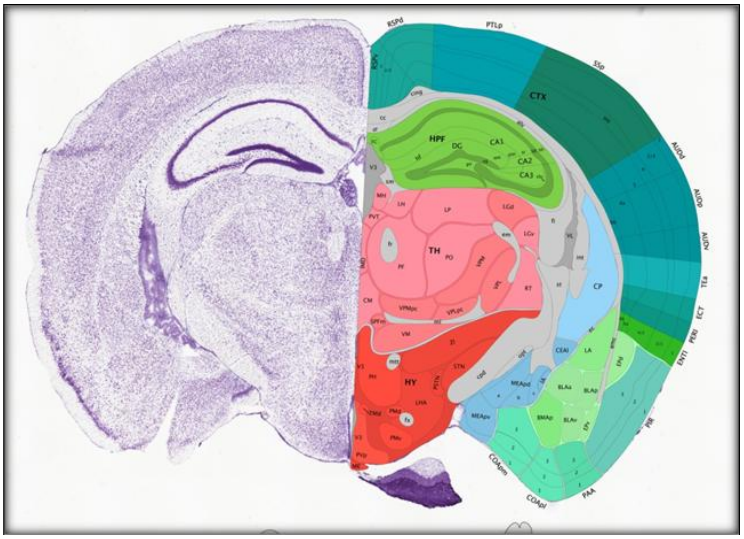
4.6. IKERKETA EREMUAK

Animalien burmuinak hiru zatitan banatu ziren (14. irudia). Aukeraturiko ikerketa eremua 5,40 mm-ko distantzia interauralan eta -3,60 mm-ko bregma distantzian kokatzen da, Paxinos eta Watsonen atlasa anatomikoa jarraituta (Paxinos eta Watson, 1982), tarteko hipokonpoko eremuari dagokiona, hain zuzen ere (15.irudia).



14. irudia: 14 eguneko arratoi baten burmuinaren bista dortsala eta tarteko hipokanpoaren argazkia. Ezkerrean, burmuineko bista dortsala, non tarteko zatia aukeratu genuen (2), gure intereseko eremuari dagokiona, alegia. Eskuman, tarteko hipokanpoaren irudi bat azaltzen, non ikerketaren eremu zehatzak azpimarratu diren.

Ikerketa gehienetarako bereziki hipokanpoko CA 1, CA 2-3 eta hortz zirkunboluzio zonaldetan (*Dentate Gyrus*, DG) eta kortex parietalean (CTX) arreta jarri genuen. Bestalde, mielinaren proteina basikoaren (*Myelin Basic Protein*, MBP) azterketarako tarteko hipokanpoko estriatum eta kanpoko kapsula zonaldetan zentratu ginen. eta neuronen konexiorako ikerketarako, aldiz, talamoa, gorputz kailukaran eta kortexean.



15. irudia: 5,40 mm-ko distantzia interauralan eta -3,60 mm-ko bregma distantzian kokatzen den tarteko hipokanpoaren irudia, non ezkerreko hemisferioan Nisslarekin tindaturiko benetako burmuin bat agertzen den eta eskumakoan zonalde desberdinak azaltzen zaizkigun. Allen Brain Atlas onlinetik aterata.

4.7. IKERKETA MORFOLOGIKOA

4.7.1. MATERIALAREN PROZESAMENDUA IKERKETA HISTOKIMIKO ETA IMMUNOHISTOKIMIKOETARAKO

Ikerketa histologiko zein immunohistokimikoetarako animaliak injekzio intraperitoneal baten bidez hil ziren, Dolethal izeneko pentobarbial sodiko eutanasikoa 20g/100 ml kontzentrazioan sartuz. Erabili beharreko bolumena animaliaren pisuaren arabera kalkulatu zen, 250 µl Dolethal animalia 100 gramoko. Animaliak intrakardiakoki perfunditu egin ziren, bonba peristaltiko baten bitartez, zeinak likido garbitzailea presioa eta fluxua konstantean pasarazten zuen. Behin animalia lokartzen genuela, bihotzeko ezkerreko aurikula mozten genion garbiketa likidoaren eta odolaren igarotzea ahalbidetuz. Lehenengo eta behin, likido garbitzailea pasarazi genuen, serum fisiologiko eta heparina %1ren arteko nahasketa batean zetzana, burmuinak garbitzeko eta aldi berean odolaren koagulazioa ekiditeko. Ondoren, finkatze-soluzioa pasarazi genuen, paraformaldehidoa %4ean, aurre-finkatzea erraztuz.

Behin paraformaldehidoarekin perfusioa bukatuta, garezurra trepanatu zen artazi eta punta laueko pintzak erabiliz burmuina ateratzeko. Garuna paraformaldehidoan mantendu zen 4 ºC-tan 24 orduz askoz jota. Finkatzeak zelula barneko elementuen insolubilizazioa ahalbidetzen du eta ehunari trinkotasuna ematen dio errazago maneiatu ahal izateko, ehunaren endekapena saihestuz.

Nissl tindaketetarako eta MBPren immunohistokimikarako, behin ehuna finkatuta genuelarik, hiru zatitan banatu eta tartekoa, hau da, tarteko hipokanpoari zegokiona, aukeratu genuen parafinan inkluitzeko. Horretarako laginak deshidratatu egin genituen goranzko graduazioa zuten alkoholetan murgilduz: alkohol 50º (90 minutu), 70º (90 minutu), 96º (90 minutuzko bi murgilketa), 100º (90 minutuzko hiru murgilketa) eta azkenik xiloletan. Deshidratazioaren ostean, laginak parafina likidoan inkluitu genituen, blokeen prestaketari ekiteko. Blokeak 1150/Acounto Reichert Jung mikrotomoaren bitartez moztu genituen, lodieraz 5 µm-ko ebakin koronalak lortuz, hauek porta gelatinizatuetan jartzen genituen eta azkenik 24 orduz 37ºC-tan mantendu genituen.

GFAP eta ionizaturiko kaltzioari lotzeko molekula moldakor 1 (*Ionized calcium binding adaptor molecule 1,* IBA-1) proteinaren immunomarkaketarako eta konexio neuronalen azterketarako, garunak paraformaldehidoan askoz jota 24 orduz finkatu ostean, sakarosa %30ean duen buffer fosfatoan (PB) 0,1M jartzen dira ehuna hondoratzen den arte, burmuina kriobabesteko helburuarekin. Lagin hauetatik 60 µmko ebakin lodiak eskuratu genituen kriotomoa (Leica 1325) erabiliz. Ebakinak azida sodikoa duen PBS 0,1M-tan jasotzen dira.

4.7.2. NISSL TINDAKETA

Tindaketa hau kresil bioleta koloratzailearen bitartez egin zen, zeina Nissl gorputzak nabaritzeko erabiltzen den. Nissl gorputzak neuronetan, erretikulu endoplasmatikoz eta polirribosoma askez eratutako gorputz pikortatu handiak dira, oso basofiloak dira RNAren edukiari esker. Hortaz, koloratzaile aniliniko basikoz intentsitate handiz markatzen dira, eta koloratzailearen pHaren eta desberdintzearen arabera Nissl gorputzak, neuronen nukleoak edo bi motetako egiturak baino ez ditugu nabarituko.

Parafinan inkluituriko ebakinak kresil bioletarekin tindatzeko, lehenengo pausua desparafinatzea izan zen horretarako xiloletan sartu ziren 10 minutuko bi bainutan, zelulen mintzak ezegonkortzeko koloratzailearen barneratzea erraztuz. Ondoren, ehuna hidratatu egiten zen graduazioan beherantz doazen alkoholetan sartuz berriro hidratatu ziren: minutu bakarreko bainu bana 100º-ko etanoletan, 96º-tan, 70º-tan eta azkenik iturriko uretan. Burmuinen ebakinak tindatzeko 57ºC-ra berotutako kresil bioletan (kresil bioletazko 0,1 g ur destilatuaren 100 ml-ko, azido azetiko glazialaren 4 tanta gehituta) murgildu genituen. Soberazko koloratzailea kentzeko ur destilatuarekin birritan garbitu ziren laginak. Ehunaren deshidrataziorako gero eta gradu altuagoko etanoletatik (50º, 70º, 96º,100º) pasatu eta azkenik xilenotan sartuko genituen 10 minutuko bi bainutan. Kontuan hartu behar izan genuen 50º, 70º eta 96º-ko alkoholek azido azetiko glaziala % 1ean izan behar zutela, desberdintze hobea erdiesteko. Azkenik DPX (Fluka, Sigma-Aldrich) erretxinaz eta porta-estalkiaz estali genituen.

4.7.3. KALTEAREN ETA TRATAMENDUAREN ERAGINKORATASUNAREN EBALUAZIO ETA KUANTIFIKAZIO MAKROSKOPIKOA

Kuantifikazio makroskopikorako, ebakin histologikotan zenbait zonaldetako argazkiak atera genituen lupa bat erabiliz (Carl Zeiss Stemi 2000-C Steromicroscope) eta hauek aztertu egin ziren ImageJ (http://imagej.nih.gov/ij/) programaz baliatuz. Kalteturiko eremuaren bi hemisferioen arteko ratioa bezala kalkulatu zen, hurrengo formulari jarraiki: ([K-I]/K) x 100, non K hemisferio kontralaterala zen eta I ipsilaterala. Emaitza kalte ipsilateralaren ehunekoa bezala adierazi zen.

4.7.4. KALTEAREN ETA TRATAMENDUAREN ERAGINKORATASUNAREN EBALUAZIO ETA KUANTIFIKAZIO MIKROSKOPIKOA

Kuantifikazio mikroskopikorako, laginak mikroskopio optiko (Olympus BX 50) batez aztertu eta argazkiak atera ziren, ondoren kalte zelularra aztertu eta kuantifikatzeko sistema neuropatologio sasi-kuantitatiboa jarraitu genuelarik (Hedtärn eta lank., 2002).

Kortex parietalaren kaltea 0 eta 4 puntu bitartean baloratu zen: 0 puntu kalterik ikusi ez zenean, puntu 1 kalteturiko zelula-talde isolatuak, 2 puntu ikusitako kaltea zenbait zelula-taldeek pairatzen zutenean, 3 puntu ehunak infartu moderatu bat agertzen zuenean eta 4 puntu kalteak ia hemisferio oso hartzen zuenean. Hipokanpoaren kasuan, CA 1, CA 2-3 eta hortz zirkunboluzio zonalde bakoitzeko puntuak gehituta kuantifikatu zen kaltea, kontuan hartutako parametroak hipotrofia (0 eta 3 puntu tartean) eta kalte zelularra (0 eta 3 puntu tartean) izan zirelarik. Beraz, hipokanporako gehinezko kalte-puntuazioa 18 izango litzateke.

Bereziki arreta jarri genion modu orokor baten infartu zistikoei eta hemisferio ipsilateraletako hipokanpoen uzkurtzeari. Zelula-kaltea ebaluatzerakoan, Nisslaren markaketa nabaria zuten zelulak aktibitate gehiago zutenak zirela onartu zen, eta bestalde, nukleoloa nabaria ez zuten zelulak kaltetutzat hartu ziren, azken hauek gainera nukleo piknotikoak azaltzen zituztelarik, hau da, nukleo osoa tindatuta zeukatenak. Kortex parietalaren eta hipokanpoaren ebaluazioaz gain, makroskopikoki ere (infartua azaltzen zuten, bi hemisferioek simetria mantentzen zuten) laginak aztertu genituen, ondorioz sistema neuropatologiko honen bitartez kalteturiko zelulek eduki zezaketen gehienezko puntuazioa 28 puntu ziren.

4.7.5. GLIAREN PROTEINA AZIDO FIBRILARRA (GFAP)

Hipoxia-iskemiak sortutako kaltearen ondorioz burmuinean glia erreaktiboaren espresioa eremuagotu egiten da eta horren ikerketarako gliaren proteina azido fibrilarraren immunomarkaketa bururu genuen. GFAP zelula gliaen zitoeskeletoaren tarteko filamentuak osatzen dituzten proteina fibrilarretako bat da, NSZan eta bereziki astrozitoetan espresatzen dena. Funtzio anitz betetzen ditu, hala nola, zelularen egituraren mantenua eta mugimendua, izan ere, burmuineko astrozitoen barne-egitura tridimentsionalaren antolakuntza babesten du, era berean kurbadura eta malgutasuna ahalbidetuz. Fluoreszentziazko ikerketa inmunohistokimikoaren bidez GFAP aztertu genuen, hau da, fluorokromoei loturik dauden antigorputz sekundarioak erabili eta mikroskopia konfokal bidez begira genituen.

Sakarosa %30ean kriobabestutako eta kriotomo bidez moztutako 60 µm-ko ebakinak aukeratu eta 12 putzutako plaka baten sartu ziren. Azida kentzeko

helburuarekin gatz fosfato tanpoian (*Phosphate Buffered Saline*, PBS) (0,1 M eta pH 7,4) 10 minutuko bi garbiketa egin genituen. PBS 0,1M garbitu eta PBS-BSA-Tw20 %1etan gehitu genuen 10 minutuz ehuna eta jarraian 5 minutuko 3 pase egin genituen blokeo-soluzioan (Triton X-100 %0.25 BSA %0.5 PBStan) mugimenduan. Ostean, laginak 1.go antigorputzarekin, anti-GFAP (monoklonala eta untxiarena, DAKO, Dinamarka) 1:1000 kontzentrazioan Triton X-100 %0.25 BSA %0.5 PBStan diluituta inkubatu ziren gau osoan 4ºC-tan ilunpean. Antigorputza ondo sartzeko ehuna iragazkortu beharra dago, horregatik detergenteak erabili egiten dira, kasu zehatz honetan Triton X-100 izan zelarik. Bestalde, BSA (*Bovine Serum Albumin*) ehuna blokeatzea ere funtsezkoa da, antigorputzek ehunarekiko duten inespezifikotasuna eragozteko, hau da, immunopositibitate faltsua ager ez dadin.

Hurrengo egunean, PBS 0,1M-rekin 10 minutuko bi garbiketa egin genituen eta 2.antigorputzarekin, Alexa Fluor 488 fluorokromodun anti-untxia (1:200, Invitrogen, The Netherlands), eta DAPIrekin (1:1000, Invitrogen) ordubeteko inkubaketa egin genuen giro-tenperaturan eta mugimenduan. Azken pausua, ebakinak porta gelatinizatuetan PBS-glizerolarekin (1:1) jarri eta estalki batekin babestu ziren. Kontrol negatiboak prozedura berdina jaso zuen lehenengo antigorputzaren inkubaketa izan ezik, eta ez zuten autofloreszentziarik adierazi. Mikroskopia konfokalaren (Olympus Fluoview FV500) bidez laginak aztertu eta argazkiak atera genituen, bereziki hipokanpoko CA1 eta hotz zirkunboluzioari arreta jarriz.

4.7.6. MIELINAREN PROTEINA BASIKOAREKIN (MBP) IMMUNOMARKATU

Mielinaren proteina basikoa, NSZko mielinadun zelulen mintz plasmatikoan azaltzen den proteina nagusia da eta MBP geneak hura kodifikatzen du. Mielina axoiaren inguruko estaldura babeslea (mielina-geruza) eratzen duen substantzia da, nerbio-bulkadak modu egokian hedatzea ahalbidetzen duena, isolatzaile elektriko moduan dihardu. MBPa oligodendrozito eta Schwann zelulen mielinaren osagairik nagusiena da, hortaz bere garrantzia NSZ-ko mielinizizazio prozesuan, izan ere oligodendrozitoen markatzaile bezala deskribatu da.

MBPren azterketarako peroxidasan oinarritzen zen sistema entzimatikoa erabili genuen, mikroskopia optikorako balio zuena. 3,3´diaminobentzidina (*3,3'-Diaminobenzidine*, DAB) kromogenoa gehitzean, hori oxidatu egiten zen antigorputz sekundarioaren peroxidasa eta hidrogeno peroxidoaren presentzian eta erreakzioaren ondorioz, argi-mikroskopioan ikus zitekeen marroi koloreko produktu bat sortzen zen. Guk peroxidasa endogenoa blokeatzen genuenez, antigorputz sekundarioaren seinalea baino ez genuen detektatzen. Izan ere, peroxidasak hidrogeno peroxidoaren deskonposizioa katalizatzen du, askatzen den oxigenoak DAB oxidatzen duelarik kolore marroi-gorrixka emanez, argi-mikroskopioan ikus daitekeena.

Lehenengo eta behin, ebakinak desparafinatzea izan zen horretarako xiloletan sartu ziren 10 minutuko bi bainutan, jarraian ehuna graduazioan beherantz zihoazen alkoholetan sartuz berriro hidratatu ziren: minutu bakarreko bainu bana 100º-ko etanoletan, 96º-tan, 70º-tan eta azkenik iturriko uretan. Peroxidasa endogenoaren iharduea blokeatu genuen 20 minututan zehar. Ondoren, 2 minututako 2 bainu PBS 0,1M erabiliz egin ziren. Ehuna iragazkortzeko tritoi x-100 PBS 0,1M-tan (Fluka, Sigma-Aldrich) 10 minutuz mantendu zen. PBS-BSA %0.5rekin 5 minutuzko bi garbiketa egin genituen, antigorputz primarioarekin laginak inkubatzeko: saguan egindako anti-MBP monoklonala, Santa Cruz Biotechnology, Dallas, Texas, AEB) 1:200 kontzentrazioan Triton X-100 %0.25 BSA %0.5 PBStan 4ºC-tan gau osoan ilunpetan.

Hurrengo egunean, PBS-BSA %0.5rekin 5 minutuzko hiru garbiketa egin genituen, bigarren antigorputzarekin laginak inkubatzeko: dioxigenina-peroxidasa loturik daukan ahuntzan egindako anti-sagua, ordu batez giro tenperaturan 1:200 kontzentrazioan PBS 0,1Mtan. Ondoren, 2 minutuko 4 garbiketa egin ziren PBS 0,1Mtan. Erreakzioa errebelatu genuen diaminobenzidina (Sigma-Aldrich) (1.25 µg DAB 50 ml PBS 0.1M eta10 µl H_2O_2 30%-ra) kromogenoa erabiliz, denbora bistaz finkatu genuelarik erreakzioak hartzen zuen kolorearen arabera, gutxi gora behera 5 eta 10 minutu bitartean. Erreakzioa gelditu eta 4 garbiketa egin ziren PBS 0,1Mtan. Azkenik, laginak Harris hematoxilinarekin kontrastatu genituen 3 segundotan sartuz, eta deshidratatu genituen graduazioan gorantz zihoazen alkoholen laguntzaz, xilenotan 5 minututako 2 bainu egin eta ebakinak porta-estalkiarekin estali genituen DPX erretxina erabiliz.

4.7.7. MBP PROTEINAREN ANALISI DENSITOMETRIKOA

Anti-MPB antigorputzarekin immunomarkaturiko laginak mikroskopio optikoz (Olympus BX 50) aztertu eta argazkiak egin genituen 40 biderreko handipenarekin. Ondoren argazkiak ImageJ (http://imagej.nih.gov/ij/) programaz baliatuz zuri-beltzeko iruditan transformatu ziren, eta bi hemisferioen arteko ratioa kalkulatu zen, hurrengo formulari jarraiki: K-I, non K hemisferio kontralaterala den eta I ipsilaterala.

4.7.8. IBA-1 PROTEINAREN IMMUNOMARKAKETA

Ionizaturiko kaltzioari lotzeko molekula moldakor 1 bereziki mikrogliak espresatzen duen proteina bat da. Bere espresioak zelula hauen aktibazioarekin bat gora egiten du, batik bat hanpadura egoeretan.

Sakarosa %30ean kriobabestutako eta kriotomo bidez moztutako 60 µm-ko ebakinak aukeratu eta 12 putzutako plaka baten sartu ziren. Azida kentzeko helburuarekin gatz fosfato tanpoian (PBS) (0,1 M eta pH 7,4) 10 minutuko bi garbiketa egin genituen. PBS 0,1M garbitu eta PBS-BSA-Tw20 %1etan gehitu genuen 10 minutuz ehuna eta jarraian 5 minutuko 3 pase egin genituen blokeo-soluzioan (Triton X-100 %0.25 BSA %0.5 PBStan) mugimenduan. Ostean, laginak 1.go antigorputzarekin, anti-IBA-1 (ahuntza, Abcam, Cambridge, Erresuma Batua) 1:1000 kontzentrazioan Triton X-100 %0.25 BSA %0.5 PBStan diluituta inkubatu ziren gau osoan 4ºC-tan ilunpean. Antigorputza ondo sartzeko ehuna iragazkortu beharra dago, horregatik detergenteak erabili egiten dira, kasu zehatz honetan Triton X-100 izan zelarik.

Hurrengo egunean, PBS 0,1M-rekin 10 minutuko bi garbiketa egin genituen eta 2.antigorputzarekin, Alexa Fluor 488 fluorokromodun anti-ahuntza (1:200, Invitrogen), eta DAPIrekin (1:1000, Invitrogen) ordubeteko inkubaketa egin genuen giro-tenperaturan eta mugimenduan. Azken pausua, ebakinak porta gelatinizatuetan PBS-glizerolarekin (1:1) jarri eta estalki batekin babestu ziren. Kontrol negatiboak prozedura berdina jaso zuen lehenengo antigorputzaren inkubaketa izan ezik, eta ez zuten autofloreszentziarik adierazi. Mikroskopia konfokalaren (Olympus Fluoview FV500) bidez laginak aztertu eta argazkiak atera genituen, bereziki hipokanpoko CA1 eta hotz zirkunboluzioari arreta jarriz.

4.8. FLUXUZKO ZITOMETRIA BIDEZKO IKERKETA

Fluxuzko zitometria analisi zelularrerako teknika oso garrantzitsu bat da, zelulen populazioak identifikatzeko tamaina eta konplexutasuna bezalako barne-parametroak erabiltzen dituena, eta era berean, propietate funtzionalak kuantifikatzea baimentzen duena fluorokromoen txertaketaren bitartez. Gainera, partikula bakoitzeko zenbait parametroen aldi bereko azterketa ahalbidetzen du.

4.8.1. ERREGISTRATURIKO SEINALEEN LORPENA ETA PROZESAMENDUA

Zelularen ezaugarri fisikoak aztertzeko batez ere hurrengo parametro hauek erabili ziren, Forward Angle Light Scatter (FALS) eta Integrated Side Scatter (ISS).

FALS zelulek eurek islatzen duten laserraren argia da, eta lagina eramaten duen kapilar likidoarekin laserraren eraso-angeluarekiko 1-10º bitarteko angeluarekin jasotzen dira. Parametroa hau zelularen tamainari dagokio, zelularen diametroarekin zuzenki erlazionaturik dagoelako eta banakako zelular agregatuetatik eta hondar zelularretatik bereiztea baimentzen du, baita zelula biziak eta hilak desberdintzea ere, azken hauek argi gutxiago barreiatzen baitute.

ISS zelula batek errefraktatzen duen argia da laserraren norabidearekiko 90º-ko angeluarekin. Zitoplasmaren isotropiaren ikasketa ahalbideratzen du, zitoplasma zenbat eta konplexuagoa izan erreflazio handiagoa dute, desbideratzen duen laserraren argiaren proportzioa handiago izango baita. Aldi berean ere zelula hilen eta bizien arteko bereiz daiteke, hilek zitoplasma oso kondentsatua eta pikortsua dutelako, seinalearen emendioa eraginez.

Ezaugarri fisiko hauetaz gai, zelulek fluxuzko zitometriaz aztertu daitezkeen propietate funtzionalak dituzte, zitometroek fluoreszentzia-seinale desberdinak aldi berean aztertzeko aukera ematen dutelako. Fluoreszentzia fenomeno fisiko bat da, non uhin luzera jakinarekin kitzikatutako substantzia batek argia bereganatzen duen eta ondoren uhin luzera handiago duen argia igortzen duen, hau da, energia gutxiago duena (Stroken legea). Zelulek fluoreszentzia beregana dezakete, parametro zelular edo

antigeno desberdinetara lotzen den fluorokromoekin inkubatu ostean. Igorritako seinale optikoak, fluoreszentziako histograma bezalako analisi sasikuantitatibozko sistemen bitartez interpreta daitezke, gure kasuan Summit v4.3 programa erabili genuen datuen azterketarako.

4.8.2. ESEKIDURA ZELULARREN ERAUZKETA

Animalien burmuinak glukosadun serum hotzarekin perfunditu ziren odola kentzeko. Garunetatik intereseko eremua aukeratu genuen, alde ipsilateralari zegokiena soilik, eta glukosadun serum beteriko petri plaketan 1 mm^3ko zatiak moztu ziren bisturiz, betiere izotzetan mantenduz. Disgregazio mekanikoz eskuratutako esekidura zelularrak nylonezko 70 µm-ko banatzaile zelularretara (BD Falcon, Becton Dickinson, Franklin Lakes, Nueva Jersey, AEB) pasa ziren eta hauek 6 putzutako plaketara (Sarstedt, Nümbrecht, Alemania), disgregazio entzimatikoarekin jarraitzeko.

Mikroplakei kolagenasa (Invitrogen) 1,5 mg/ml-ko kontzentrazioan Hanks (*Hank´s Balanced Salt Solution*, Sigma) kultibo-medioan diluituta zegoen 4 ml-ko disoluzioa gehitu zitzaien eta 20 minutuz 37ºC-tan agitazioan mantendu ziren. Jarraian, esekidura zelularrei beste 4 ml Hanks gehitu eta zentrifugatu (Labogue 400, Heraeus) egin ziren 1500 rpm-ko abiaduraz 5 minutuz. Gainjalkina bota ostean, pelleta Hanks kultibo-mediozko 5 ml-tan birsuspenditu zen. Fluxuzko zitometriaz egindako ikerketarako erabilitako esekidura zelularren kontzentrazioa 10^6 zelula/ml izan zen. Zenbait parametro zelular aztertu genituen 4 fluorokromo desberdin erabiliz (2.taula).

2. taula: Erabilitako fluorokromoen laburpen taula, haien kitzikapen eta igorpen uhin luzerak eta kontzentrazioak azaltzen direlarik.

FLUOROKROMOA	KITZIKAPENA (nm)	IGORPENA (nm)	KONTZENTRAZIOA
Rh 123	507	529	4 µl / 100 µl
DCFH-DA	504	529	4 µl / 100 µl
NAO	595	519	4 µl / 100 µl

4.8.3. MITOKONDRIAREN BARNE-MINTZAREN OSOTASUNAREN AZTERKETA

Kardiolipinak, mitokondriaren barneko mintza osatzen duten fosfolipido nagusia, honen egonkortasuna mantentzen laguntzen du. 10-N-nonyl-acridin orange (NAO, Invitrogen) fluorokromoak kardiolipinara batzeko joera du, zelulen mitokondrien egoeraren berri ematen duelarik.

Etanol 70º-rekin fixaturiko esekidura zelularretik (10^6 zelula/ml) 750 µl PBS-arekin garbitu ziren zentrifugatuz 1500 rpmko abiaduraz 5 minutuz, eta eskuratutako pelleta NAO (10^{-2} M) 4 µl-rekin inkubatu zen 30 minutuz 37ºC-tan. Soberazko NAO bi garbiketekin deuseztatu zen eta fluoreszentzia zitometriaz aztertu zen.

4.8.4. MITOKONDRIAREN MINTZAREN POTENTZIALAREN NEURKETA

Mitokondriaren mintz-potentziala aztertu zen Rhodamina 123 (Rh 123, Invitrogen) fluorokromo kationiko lipofilikoa erabiliz, hauxe bizirik dauden zelulen mitokondriaren barnealdean metatzen da mintzaren potentzialaren intentsitatearekiko proportzionalki. Mitokondriaren aktibitatea mitokondriaren barne-mintzaren bi alboetako potentzial desberdintasunean islatzen da, bi alboetako kargen banaketa asimetrikoaren ondorio dena, eta ATParen sintesirako funtsezkoa. Mitokondriaren mintz potentziaren neurketak mitokondriaren ekintza metabolikoa aztertzea baimendu zigun, honen galera apoptosiaren ezagugarria baita.

Horrela, esekidura zelularretik (10^6 zelula/ml) 750 µl inkubatu ziren Hanksen diluituriko Rh 123 (4 µl/100 µl) fluorokromoarekin 30 minutuz 37ºCtan. Denbora honen ostean, zelulak Hanks kultibo-medio isosmolarraren 1 ml-rekin garbitu ziren, birritan zentrifugatuz 1500 rpm-ko abiaduraz 5 minutuz, gainjalkina deuseztatuz. Bi garbiketen ostean, mitokondrietan metatu ez den soberazko Rh 123 kentzeko, zelulen esekidurak 30 minutuz Hanksen inkubatu ziren 37ºC-tan ere. Azkenik, bi garbiketen ondoren, pelletak 350 µl Hanksen birsuspenditu eta zitometrotik pasa ziren.

4.8.5. OXIGENOAREN ERRADIKAL ASKEEN EKOIZPENA

Oxigenoaren erradikal askeen ekoizpena kuantifikatzeko oxidaziora sentikorra den 2,7-dichlorodihydeofluorescein diacetate (DCFH-DA, Invitrogen) fluorokromoa erabili genuen. –DA erradikalaren konfigurazioak zitoplasman pasiboki eranstea baimentzen du. Behin erantsita, diazetato taldeek askatu egiten dira zelulen barne-esterasen ekintzen eraginez, DCFH azalduz. DCFHa konposatu polarra da, zelularen barnean bahituta geratzen dena eta DCF molekula fluoreszentea bilaka daitekeena, zelularen oxigeno peroxidoek hidrogenioiak galtzarazten badiote.

Ehunetatik lortutako esekidura zelularretik (10^6 zelula/ml) 750 µl garbitu ziren 5 minutuz 1500 rpm-ko abiaduraz zentrifugatuz. Eskuratutako pelleta Hanksen diluituriko DCFH-DArekin (4 µl/100 µl) birsuspenditu eta 30 minutuz 37ºCtan inkubatu zen. Azkenik, bi garbiketen ondoren, pelletak 350 µl Hanksekin birsuspenditu ziren, fluxuzko zitometroz aztertzeko.

4.9. DENBORA ERREALEKO POLIMERASAREN KATE-ERREAKZIO KUANTITATIBOAREN BIDEZKO ESPRESIO GENIKOAREN AZTERKETA

Denbora errealean buruturiko polimerasaren kate-erreakzio (PCR) kuantitatiboaren bidez (*Real Time Quantitative Polymerase Chain Reaction*, RT-qPCR) gure intereseko zenbait geneen espresioa aztertuko genuen. RT-qPCRa PCR estandarraren eraldaketa bat da eta lagin baten azido erribonukleikoaren mezularia (*Messenger Ribonucleic Acid*, mRNA) eta DNA kuantifikatzeko erabiltzen da.

Gure kasuan mRNA mezulariaren espresioa ikertu nahi izan genuenez, lehenengo eta behin mRNAtik DNA osagarria (*Complementary Deoxyribonucleic acid* cDNA) sortu genuen, alderantzizko transkripzio prozesuari esker (*Retro-Transcription*,RT). Orduan, anplifikatu nahi genituen geneen sekuentzia-tartea mugatzen zuten primerrak erabiliz, PCRa egin genuen.

Fluorokromoak (EvaGreen) gehituz denbora errealean egindako PCR kuantitatiboa burutu genuen, zeina fluoreszentziaren detekzioan oinarritzen den. Fluoreszentziazko seinalea proportzionalki areagotzen da PCR ziklo bakoitzean sortutako produktu kantitatearekiko. Hori dela eta, gai izan ginen bukaeran kuantifikazio erlatibo bat egiteko, gure intereseko geneen espresioa erreferentziazko gene batzuen espresioarekin alderatzean.

4.9.1. RNA TOTALAREN ERAUZKETA

Zazpi eguneko arratoi neonatalak pentobarbital sodiko eutanasikoa erabiliz hil ziren, hipoxia-iskemia eragin eta 0, 3 eta 12 ordutara. Behin animalia anestesiatuta zegoela, serum fisiologikoarekin perfunditu eta burmuina atera genuen. Ikerketarako erabilitako ehuna tarteko hipokanpoari zegokion alde ipsilaterala izan zen, momentu oro izotzetan mantendu genuena. Gure ikerketarako ehunaren RNA totala RNA Mini Kit (Ambion, Life Technologies) kit komertziala erabiliz erauzi genuen, RNA purifikatzeko silizezko zutabetan oinarritzen zena. Lehenengo pausua, lagina eppendorfetara pasatzea eta horiei 0.6 mL Lysis Buffer eta 2-mercaptoetanolaren arteko nahasketa gehitzea izan zen, jarraian rotor-stato homogenizatore batekin laginak 45 segundoz homogenizatu ziren eta 5 minutuz zentrifugatu genituen (26000 x g). Hemendik aurrera RNA Mini Kitaren argibideak jarraitu genituen, RNA totala erauzteko.

4.9.2. ERAUZITAKO RNA TOTALAREN KUANTIFIKAZIOA ETA KALITATEAREN EGIAZTATZEA

Gure laginetako RNA totalaren kantitatea ezagutzeko Multi-Mode Microplate Reader (Biotek Instruments, INC Winooski, Vermont, USA) erabili genuen. Lagineko 1µl-tik abiatuz RNA kontzentrazioa, ng/ µl bezala adierazita, lortu genuen. Absorbantzia 230 nm (EDTA, karbohidratoak, fenolak), 260 nm (RNA) eta 280 nm (proteina) uhin-luzeratan neurtu genuen, RNasa ez zuen ura zuri moduan erabiliz, horrela A260/280 eta A260/230 ratioak eskuratu genituelarik.

A260/280 eta A260/230 ratioak RNAren purutasunaren adierazleak dira, RNA-puntuaren absorbantziak proteina puntuaren bikoitza izan behar du gutxi gora-behera. Beraz, laginen ratioak 1,8 eta 2 bitartean badaude, purutasun nahikoa dutela, hots, erauzketa eta purifikazio prozesuen ondoren lagina hondar organiko ala inorganikoz ezta proteinez kutsatuta ez dagoela, onartzen da. RNA total hau -80°C-tan gordetzen da erabili arte.

4.9.3. cDNA SINTESIA

cDNAren sintesirako Fluidigm Reverse Transcription Master Mix kita erabili zen, gomendaturiko argibideak jarraiki. Horretarako 1µl RNA erabili zen 5µl alderantzizko transkripzio (RT) erreakzio bolumenean (reverse transcription master mix 1 µl, RNasarik ez duen ur 3 µl). Alderantzizko transkripzioaren erreakziorako, laginaren RNA master mixarekin inkubatu zen lehenengo 25ºC-tan 5 minutuz eta gero 42ºC-tan 30 minutuz, eta azkenik alderantzizko transkriptasa entzima inaktibatu zen bero txoke baten bitartez, 85ºC-tan 5 minutuz.

4.9.4. RT-qPCR BIDEZKO ESPRESIOAREN ANALISIA

RT-qPCR bidezkoa espresio genikoaren analisia UPV/EHU-ko Ikerkuntzarako Zerbitzu Orokorretako (SGIKER) Genomika Zerbitzuak burutu zuen. Proiektua mRNA-ren espresioaren azterketan zetzan, horretarako Fludigm-eko BioMark HD Nanofluidic qPCR System teknologia GE 96.96 Dynamic Arrays IFC-rekin konbinatu zen.

Prozedurarako ADP 37, Fast Gene Expression Analysis Using EvaGreen on the BioMark or BioMark HD System, Advanced Development Protocol 37 (PN 100-3488, Rev.D1) protokoloa jarraitu zen. Erreakziorako Master Mix SsoFastTM EvaGreen® Supermix with Low ROX, Bio-Rad Laboratories, PN 172-5211 erabili zen. Azkenik, mRNAren detekziorako aurrediseinaturik dauden primerrak, PrimeTime qPCR predesigned primers (IDT Integrated DNA Technologies), erabili genituen, zeinak EvaGreen bidezko anplifikazio eta detekziorako espezifikoak diren primer pareengatik osatutik dauden (100 mM) (3. eta 4. taulak).

3. taula: Erreferentzia gene hautagaiak (barne-kontrolak) eta bere primerren erreferentziak.

	ERREFERENTZIA-GENE HAUTAGAIAK	ERREFERENTZIA
1	**B2M**	Rn.PT.39a.22214834
2	**PPIA**	Rn.PT.39a.22214830
3	**TP**	Rn.PT.58.5097819
4	**HRPT**	Rn.PT.58.37395539
5	**PGK1**	Rn.PT.58.36893909
6	**GAPDH**	Rn.PT.58.35727291
7	**RPLP0**	Rn.PT.58.45174577

4.taula: Ikerketarako aukeraturiko 20 geneak dagokien ikurrekin, eta mRNAen detekziorako PrimeTime qPCR predesigned primers (IDT Integrated DNA Technologies) erabilitako primerren erreferentzia eta deskribapenekin.

	ITU-GENEA	IKURRA	ERREFERENTZIA	DESKRIBAPENA
1	C-fos	***Fos***	68033618	Rn.PT.58.13000819
2	Egr-1	***Egr1***	68033624	Rn.PT.58.35398845
3	TNF-alpha	***Tnf***	68033645	Rn.PT.58.11142874
4	HSF 1	***Hsf1***	68033648	Rn.PT.58.36380980
5	COX 2	***Ptgs2***	68033627	Rn.PT.58.9481136
6	NOS 2 (Inos)	***Nos2***	68033630	Rn.PT.58.9699876
7	SOD	***Sod2***	68033633	Rn.PT.58.7509049
8	HIF 1 alpha	***Hif1a***	68033651	Rn.PT.58.12503723
9	Caspase 3	***Casp3***	68033621	Rn.PT.58.13133040
10	Bax	***Bax***	68033636	Rn.PT.58.44501421
11	Bcl-2	***Bcl2***	68033639	Rn.PT.58.7362966
12	GFAP	***Gfap***	68033719	Rn.PT.58.38023396
13	Vimentin	***Vim***	68033654	Rn.PT.58.36145160
14	NG2	***Cspg4***	68033642	Rn.PT.58.18459521
15	Synapthophysin	***Syp***	68033660	Rn.PT.58.37045062
16	Spinophilin	***Ppp1r9b***	68033657	Rn.PT.58.44155054
17	IL6	***Il6***	68033663	Rn.PT.58.44400168
18	IL10	***Il10***	68033722	Rn.PT.58.13840513
19	VEGF	***Vegfa***	68033725	Rn.PT.58.9773600
20	Cytochrome c, somatic	***Cysc***	68033716	Rn.PT.58.34830017

4.9.5. SPECIFIC TARGET AMPLIFICATION (STA)

PCR kuantitatiborako cDNAzko 5 µl beharrezkoak izan ziren, Dynamic Array IFC-ren formatuaren arabera 9 ala 6,7 nl-ko 48 ala 96ko erreakzio-ganberatan banandu zirenak. Bolumen hain txikia zela eta, detekziorako gutxienez genearen 500-1000 kopia behar izan ziren. Geneen espresioa aldakorra denez, tarteko espresioa duten eta espresio baxua duten geneentzat gutxienezko 500-1000 kopietara heltzen ez direnez, PCR kuantitatiboa baino lehenagoko cDNAren aurreko anplifikazio bat egin zen. Aurre-anplifikazio erreakzio hau ziklo gutxiko PCR batean oinarritu zen, ikertu beharreko entseguen pool batetik abiatuz, hortaz ikertu beharreko geneen kopia kopuru espezifikoa ezagutzea funtsezkoa izan zen. Aztertuko ziren entsegu guztiak, erreferentzia-geneak barne, gehitu izan ziren. Honek RNA kantitate txikiago batetik abiatzea baimendu zigun cDNA sintesirako (10-100 ng), lagin bakoitzeko kantitatean aurrezpena suposatuz. Aurre-anplifikaziorako QIAGEN Multiplex PCR Kit (Cat N. 206143) kita erabili genuen.

27 primer mixen pool batetik abiatu ginen, 500 nM (10X) kontzentrazioan (1/200) TE-en diluituta (10 mM Tris, pH 8.0, 0,1 mM EDTA). Aurre-anplifikazio erreakzio bakoitzeko erabilitako bolumenak honako hauek izan ziren, bukaera bolumena 5 µl izan zirelarik: QIAGEN Multiplex PCR Master Mix-aren 2.5 µl (2x), entseguen poolen 0.5 µl (10X) (erreakzioaren bukaeran 50 nM), cDNA-ren 1.25 µl. Aurre-anplifikaziorako laginak 95ºC-tan 15 minutuz mantendu ziren, eta gero 95ºC-tan 15 segundoko eta 60ºC-tan 4 minutuzko 14 ziklo jasan zituzten.

Aurre-anplifikazioaren ostean, laginak Exonucleasa I-rekin (*E. coli*) (New England Biolabs, Cat N. M0293L) tratatu ziren erantsi ez diren primerrak kentzeko. eLagin bakoitzeko exonuclease I premixa, ur ultrapuruaren 1.4 µl, Exonuclease I Reaction Bufferraren 0.2 µl eta Exonuclease I-aren (20 units/µl) 0.2 µl izan ziren. STAaren 5 µl-etara gehitu beharreko bolumena beraz 2 µl izan zen, eta ondorioz, bukaerako bolumena STA eta Exo I gehituta 7µl izan zen. Liseriketarako laginak 37ºC-tan mantendu ziren 30 minutuz eta Exo I-ren inaktibaziorako 80ºC-tan 15 minutuz. Bukatzeko aurre-anplifikaturiko cDNA EDTA baxuko TE-tan diluitu ziren (1:10).

4.9.6. qPCR-REN PROTOKOLOA

Ikerketa-denbora bakoitzeko (0 h, 3 h, 12 h) lagin estandarrak migratu ziren, RNA pooletik lortutako STA-tik (aurre-anplifikaturiko cDNA) abiatutako segida diluzioak, hain zuzen ere. 6 segida diluzio edo lagin estandar migratu ziren, 1/5-eko diluzio faktore batekin. Primerren bukaerako kontzentrazioa qPCR erreakzioa 500 nM-koa izan zen, bai forwrd zein reverserako. Geneen hiru erreplika egin ziren, erreplika teknikoak deritzenak, 5. taulan azaltzen diren pausuak jarraituz.

5. taula: RT-qPCR-rako protokoloaren pausuak.

Pausuak	Tenperatura eta iraupena
1.go pausua: Thermal Mix (Eva Green-rako espezifikoa dena)	70ºC, 40 minutu. Ramp rate 5.5ºC/s
	60ºC, 30 segundo. Ramp rate 5.5ºC/s
2. pausua: Hot Start/Aktibazioa	95ºC, 1 minutu. Ramp rate 5.5ºC/s
3. pausua: 35 ziklo/Anplifikazioa	96ºC, 5 segundo. Ramp rate 5.5ºC/s 60ºC, 20 segundo. Ramp rate 5.5ºC/s
4. pausua: Melting Curve	60ºC, 3 segundo, Ramp rate 1ºC/s
	60ºC-95ºC Ramp rate 1ºC/ 3 s

4.9.7. ESPRESIO GENIKOAREN EMAITZEN ANALISIA

Fluidigm Real-Time PCR Analysis Software version 3.1.3. izan zen erabili genuen programa anplifikazio-grafiken eta desnaturalizazio-kurben analisirako eta Ct (raw data, input data for analysis) balioak eskuratzeko. Programak Ct balioak lortu eta emaitzak HeatMaps-etan adierazi zituen, non Ct-ren balioa kolore-eskalatan azaldu zen. Ct-en ondorengo analisietarako edo datu gordinen analisirako (QC, efizientziaren zuzenketa, lagin bakoitzeko bataz besteko Ct-aren kalkulua, normalizazioa, RQ-ren kalkulua, analisi estatistikoa eta Fold Change) GenEx (MultiD) programa erabili genuen.

Lehenengo eta behin, gene bakoitzaren RT-qPCR-ren efizientzia, Ct vs zegokion lagin estandarraren DNA kontzentrazioaren adierazpen grafikotik ateratako zuzenaren maldatik abiatuz kalkulatu zen. Ordu desberdinetako laginak Fluidigmen run edo txip desberdinetan migratu zirenez, ikerketarako lagin eta lagin estandarrez gain bi lagin gehigarri ere migratu ziren, inter-run kontrolak ala calibrator (IRC) deiturikoak, txip desberdinen arteko bariazioak normalizatzeko. STA lagin berbera izan zen hiru txipetan migratu genuena (pooletik lortutako STA gehigarria). Gainera, 12 kontrol negatibo (No Template Controls ala NTCs) migratu ziren, primerren dimerorik detektatu ez genuelarik 27 genetan. Gainera, disoziazio-kurba arraroak (gainontzeko erreplika eta taldeko laginengandik desberdinak zirenak) zituzten erreplikak ez genituen kontuan hartu.

RT-qPCR bidezko espresio genikoaren kuantifikazioa erlatiboa da, lagin desberdinen artean itu-genearen espresioa erreferentzia-genearekiko konparatzen delarik. Erreferentzia-genea esperimentuaren baldintzetan aldaketarik jasaten ez duen genea da, barne-kontrol moduan erabiltzen dena, honi itu-gene zehatza baten espresioaren normalizazioa deritzolarik. Honetaz gain, laginen RNA totalaren kontzentrazio desberdinekiko normalizatzen da, barne-kontrolen kantitatea aldatzen bada cDNA sintesirako erabilitako RNA kantitate desberdinagatik izan beharko litzateke, eta ez bere espresio aldaketengatik. Ez dagoenez gene bakarra baldintza guztietan bere espresioa berdin mantentzen duenik, 7 erreferentzia-gene aztertu genituen egokiena zein izan zitekeen baloratzeko. Horretarako Norfinder eta Genorm programak erabili genituen, zeinek algoritmotan oinarrituta erreferentzia-gene hautagaien artean onena zein den iradokitzen duten.

Kuantifikaziorako bi metodo nagusi daude, itu-genearen eta erreferentzia-genearen anplifikazioen efizientziak konparagarriak diren arabera. Guk ΔΔCt metodoa efizientziaren zuzenketarekin aukeratu genuen, ikerturiko geneen Ct-ak lagin bakoitzerako aukeraturiko erreferentziazko geneen bataz bestekoarekin normalizatu genuen (ΔCt) eta ondoren HI eta trataturiko animalien ΔCt kontrolekin konparatu genituen. Azkenik, ΔΔCt lortu dugunean, logaritmoetara pasatzen da eta eskuratzen dugun azken balio horri Fold Change deritzo, hots, itu-genea kontrolarekiko zenbat bider areagotzen edo gutxitzen den, eta hauxe estatistiko esangarria den edo ez aztertuko dugu.

4.10. PORTAERAREN AZTERKETA

Portaeraren ikerketak Euskal Herriko Unibertsitateko Medikuntza eta Odontologia Fakultateko Farmakologiako Saileko Leyre Urigüen Echevarria irakaslearen laguntza eta gainbegiratzearekin burutu egin ziren. Portaera testak animaliak helduak zirenean, zehazki 90 egun zituztenean (P90) ebaluatu ziren modu itsu batean (6.taula). Helburua entzefalopatia hipoxiko-iskemikoak kognizioan zein modutan eragiten zuen eta tratamenduek efektu onuragarriak zituzten aztertzea izan zen.

Animalien portaera ikertzerakoan ezinbestekoa da probak egiten diren bitartean ahalik eta giroko aldaketarik gutxien egotea, izan ere animaliek edozein aldaketa sumatzen dutenean portaeran eragina izango du. Hortaz, animaliategiaren baldintzak konstanteak mantendu behar dira beti, tenperatura besteak beste, eta 12 ordutako argi-zikloak ere errespetatuz. Gainera, probak egun desberdinetan egin zirenean, ordu berdinean egin ziren, eguneko argia eta arratoien barne-zikloa berdina izateko, orokorrean goizeko lehenengo ordutan burutu genituelarik. Mantentze-garaian kutxa bakoitzean 5 animalia baino gehiago ez genituen izan, arrak eta emeak bereizi genituelarik. Frogen aurretik animaliek ikertzailea ezagutzea garrantzitsua. Horregatik, frogaren egunean, animaliak gelara gutxienez 30 minutu lehenago eraman genituen egoera berrira ohitu zitezen.

6. taula: Portaera testentzako erabilitako animalia kopurua 64 izan zen, zeinetatik 35 arrak ziren eta 29 emeak.

	KONTROLA	HI	RVT	DHA	RVT+DHA
Arrak	8	9	4	8	0
Emeak	9	5	6	6	9
Guztira	17	14	10	14	9

4.10.1. EREMU IREKIAREN TESTA

Aktibitate lokomotor orokorra eta antsietatea neurtzeko eremu irekiaren testa erabili genuen, laukizuzen (60 x 60 x 30 cm) itxurako kutxan zetzana. Zurezko kutxa zuria zen eta beheko hondoa beltza zuen, arratoiak bereizi ahal izateko. Zorua 16 laukitan banatuta zegoen, erdiko aldeari lau zegokien eta gainontzeko 12 kanpoko aldeari edo periferiari (16.irudia).

Esperimentuak hasi aurretik, gela isil eta argi konstantearekin (300 lux) burutu zirenak, animaliak 30 minutuz gelako girora ohitu ziren. Arratoiak banan-banan kutxaren erdialdera sartu ziren eta 30 minutuz bideo kamera baten bidez haien portaera grabatu egin zen. Ondoren, grabaketak SMART (Spontaneous Motor Ativity Recording and Tracking) programaren 3.0 bertsioaren (Panlab, Bartzelona) bitartez aztertu egin ziren, animaliek erdialdean, kanpoaldean eta guztira pasatako denbora (s), distantzia (mm) eta batezbesteko abiadura (s/mm) neurtu genituelarik. Beraz, lomozio-aktibitate orokorraz gain, froga honek erdiguneko edo kanpoaldeko lokomozio-aktibitatea bereizten lagundu zigun, antsietate maila neurtzeko balio duelako erdigunea antsiogenikoagoa baita.

16. irudia: Eremu irekiaren testaren argazkia, non arratoiei libreki ibiltzeko askatasuna ematen zitzaien 30 minutuz, eta ostean haien aktibitate lokomotorra zenbait parametroen arabera ebaluatu egiten zen.

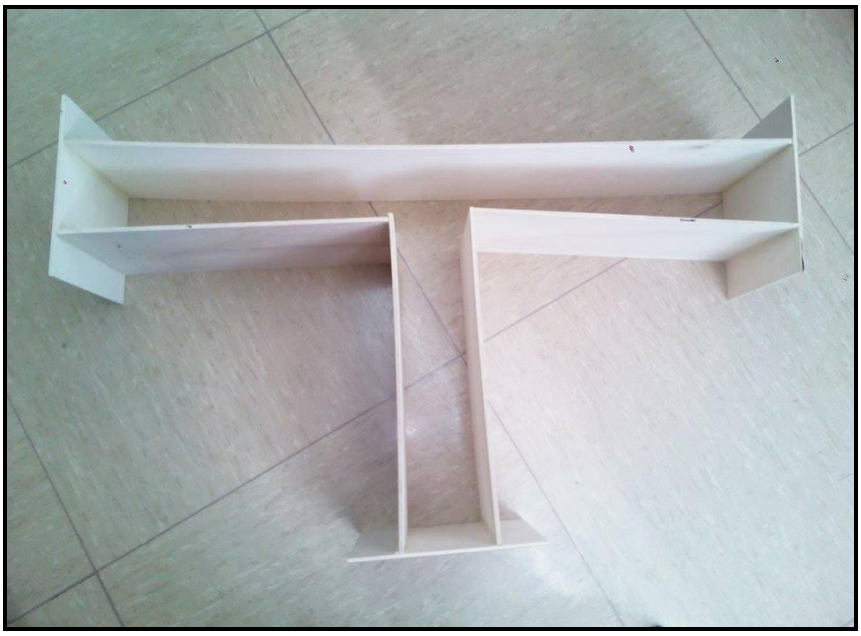
4.10.2. PLATAFORMA ZULATUAREN FROGA

Froga hau animalien aktibitate miatzailea, antsietatea eta neofobia aztertzeko diseinatuta dago. Plataforma zulatuak 5 cm-ko diametroko 16 zulo zituen taula batek osatzen zuen, kutxa batean sartuta (62x62x36 zm) zegoena, hau da pareta eta sabaiez estalita zegoen, animalia ilunpean gordetzen zelarik. Plataformak kutxaren oinarritik 7 cm-ko altueran zegoen eta beheko aldea ireki zitekeen, modu horretan, ikertzaileak animaliak burua zuloetatik erakusten zuenean bakarrik ikustea baimenduz. Esperimentuak hasi aurretik, gela isil eta argi konstantearekin (300 lux) burutu zirenak, animaliak 30 minutuz gelako girora ohitu ziren. Arratoiak banan-banan kutxaren erdialdera sartu ziren eta 5 minutuko saio bakarrean miaketa kopurura zenbatu, hots, zuloetan burua begien mailaraino zenbat bider sartzen zuten, eta zuloak zenbat denboratan esploratzen zuten neurtu genuen.

4.10.3. T ITXURAKO LABIRINTOAREN TESTA

Arratoien epe luzeko memoria espazialaren azterketa T itxurako labirinto bat erabiliz burutu zen. Froga honek hipokanpoaren osotasuna ikertzen du, izan ere, ingurumenean ez galtzeko gizakiok eta animaliek mapa kognitiboak egiten ditugu kanpoko seinalez baliatuz. Hau da, oroimen ikus-espazialari daukagu, baina hipokanpoa urratuta egon ezean oroimen hau ezin da sortu ezta erabili ere. Labirintoak 31 cm-ko beso nagusi bat eta alboetan 31 cm-ko beste bi beso zituen, denek altueraz 23 cm eta zabaleraz 18 cm zituztelarik. Alboetako besoak atea mugikorren bitartez beso nagusitik bananduta zeuden (17.irudia).

Ohitzearen aurreko astetik animaliei janaria murriztu zitzaien, eme bakoitzak 15 g janari jasotzen zituen bitartean arrek 20 g jasotzen zituzten, eta baldintza hauek mantendu ziren arratoiek esperimentua bukatu arte. Hortaz, animalia bakoitzari bere gorputz pisuari zegokion janari kopurua %85-ra gutxitu zitzaion. Arratoi desberdinen arteko testen artean labirintoa garbitu egin zen, baina ez saio bakoitzaren artean. Testaren ordainsaria janaria zen, 5 gramo hain zuzen ere. Esperimentu osoa hiru partetan zetzan, ohitzea, entrenamendua eta azkeneko proba. Esperimentuak hasi aurretik, gela isil eta argi konstantearekin (300 lux) burutu zirenak, animaliak 30 minutuz gelako girora ohitu ziren.



17.irudia: T itxurako labirintoa, non arratoien memoria espaziala aztertu genuen.

Ohitze fasean zehar, animalia guztiak T itxurako labirintoan sartu ziren eta 90 segundoz utzi zitzaien. Prozedura hau hirutan errepikatu genuen 5 egunetan. Entrenamenduan zehar, animalia guztiek sei saio burutu zituzten egunero. Saio bakoitza bi lasterketetan zetzan: behartutakoa eta askea. Behartutako lasterketan, arratoiak labirintoko zabaldurik zegoen besoan janaria eskuratzeko behartu ziren, beste besoa atea mugikorren bitartez itxita zegoelarik. Animaliak hasierako puntura bueltatu zitzaien 10 segundoren ostean. Lasterketa askean, animaliak modu askean ibiltzeko eta nahi zuten besoa hautatzeko aukera eman zitzaien. Arratoiek aurretik beharturiko besoaren kontrako besoa aukeratuz gero, janaria jasotzen zuten sari moduan, baina beso berbera hautatuz gero, aldiz, ez zuten ordainsaririk erdiesten. Saio bakoitzaren artean bost minutuko atsedenaldia eduki zuten.

Entrenamenduaren fasean bukatutzat hartu genuen animalia kontrolek %70 baino asmatze gehiago lortu zituztenean bi egun jarraituetan zehar. Testaren egunean, arratoiek hiru txanda egin zituzten zeinetan behartutako lasterketen arteko tartea 10 segundokoa zen, eta beste hiru non atsedenaldia 40 segundokoa zen. Egunaren arabera janaria kokatzeko besoa eta tartearen denbora zorizkoa izan zen, baldintza bakar bat mantenduz, kokalekua eta tartea ezin zirela berdinak izan hiru lasterketa jarraituetan. T itxurako labirintoaren testan, 10 eta 40 segundoren ostean bide egokia zenbat aldiz aukeratu zuten neurtu genuen, hots, besoan lau hankak sartzen zituztenean hautatu egiten zutelaren seinale onartzen genuelarik.

4.10.4. OBJEKTU BERRIAREN MIAKETA TESTA

Objektu berriaren miaketa testaren bitartez epe luzeko landutako memoria balioztatu genuen, ordainsaririk erabili gabe. Funtzio kognitiboak garun-azalean eta hipokanpoan finkatzen dira, hortaz garunaren atal horien funtzionamendua ikertzeko froga hau burutu genuen. Esperimentuak hasi aurretik, gela isil eta argi konstantearekin (300 lux) burutu zirenak, animaliak 30 minutuz gelako girora ohitu ziren. Lehenengo egunean arratoiak kutxara (15x28x50 cm) ohitu egin ziren 10 minutuz, eta hurrengo egunetan kolorez eta itxuraz berdinak ziren bi objektu (objektu familiarra) miatzeko aukera eman zitzaien 10 minutuz. Azkenean, probaren egunena objektu familiar horietako bat berri batez ordezkatu genuen, objektu honek ezaugarri desberdinak zituelarik, eta era berean 10 minutuz miatzen utzi genuen. Testan animaliek objektu berrian egondako denbora eta objektu ezagunean egondakoa neurtu genuen kronometro baten bidez, eta horretaz gain haien arteko ratio bat kalkulatu genuen ([objektu familiarrean egondako denbora ken berrian egondakoa] / [bi objektutan guztira egondako denbora]), eta ratio hau epe luzeko memoriaren adierazgarri gisa onartu genuen.

4.11. KONEXIO NEURONALEN AZTERKETA

Nerbio-sisteman barreneko konexio neuronalak aztertzeko urtetan zehar teknika desberdinak erabili izan badira ere, gaur egun trazatzailen metodoa da arrakasta gehien duena. Neuronetako axoien ibilbidea ikertzeko, axoien fluxuaren ikasketetan oinarrituta dagoen metodo hau erabiltzen da, izan ere markatzaile edo trazatzaileak zelulek zurgatzen dituzten gai kimikoak dira garraio aktiboaren bitartez luzapenetatik garraiatuak izaten direnak.

Trazatzaileak aurrerakoiak izan daitezke, somatik axonetara garraiatuak izaten direnak; beste batzuk atzerakoiak, kontrako bidea egiten dutenak, hots, axonetatik somara; edo bi ibilbideak egin ditzaketenak, aurrerakoiak zein atzerakoiak izan daitezkeenak, hain zuzen ere.

Neuronetako axoien ibilbidea ikertzeko molekula markatzailea ikasi nahi dugun zonaldean injektatu behar da animalia anestesiatuta mantentzen den bitartean. Gero, trazatzailea denbora batez garraiatzeko denbora uzten da, ibili behar duen distantzia egiteko axonetan zehar. Denbora horren ostean, animalia hil eta burmuina aztertzen da, jatorrizko zelulak zeintzuk diren, markaturiko axoien ibilbidea zein den eta proiekzioaren itua zein den zehazteko.

Detekziorako metodoa trazatzailearen araberako izango da, gure kasuan trazatzaileen aurkako antigorputzez baliatu ginen immunotindaketak egiteko. Trazatzaileen metodoan funtsezkoa den pausua trazatzailearen injekzioa zehaztasun eta abileziaz egitea da, geroko detekzioa egokia izateko. Azkenik, injekzioaren osteko denbora optimoena zein den ezagutu behar dugu, denboraldi laburregiak markaketa osatugabea ekar dezakeen bitartean luzeegiak markaketa inespezifikoa eman dezake.

4.11.1. TRAZATZAILEEN INJEKZIOA

Trazatzaileen ikerketak Euskal Herriko Unibertsitateko Medikuntza eta Odontologia Fakultateko Neurozientzietako Saileko Luis Martínez Millán irakasle emerituaren laguntza eta gainbegiratzearekin burutu egin ziren. Konexio neuronalen ikerketa portaera testak egiteko animalia berdinekin burutu ziren, beraz helduak zirenean, gutxi gora-behera 100 egun zituztenean (P100).

Aurre-trazatzailea BDA (*Biotinylated Dextran Amines*) (Invitrogen) injektatu egin zen (2 µl) kortexean, 5,40 mm-ko distantzia interaurala eta -3,60 mm-ko bregma distantzian, estereoktatiko baten bidez egiten da animaliak anestesiatuta zeudelarik.

4.11.2. LAGINEN ESKURAKETA

Trazatzaileen azterketarako animaliak pentobarbital sodiko eutanasiko batekin hil ziren, trazatzaile aurrerakoia injektatu eta aste baten ostean, animaliak gutxi gora-behera 100 egun zituztenean. Paraformaldehido %4 hotzarekin perfunditu ostean, laginak finkatu, kriobabestu eta kriotomo bidez tarteko hipokanpoari zegokien 60 µm-ko ebakinak eskuratu genituen.

4.11.3. TRAZATZAILE AURRERAKOIAREN DETEKZIOA: BDA

Behin ebakinak aukeratu eta plaketan genituelarik, ur oxigenatuan 1:29 kontzentrazioan PBSan (0,1 M pH 7,4) 30 minutuz mantendu ziren inguru tenperaturan. PBSan (0,1 M pH 7,4) 5 minutuko 3 garbiketen ostean, Abidin-biotina konplexuarekin (ABC) (Innovaboscence) PBS-Tx-an (0,1 M pH 7,4) inkubatu ziren, 1:200 diluituta, 24 orduz 4ºC-tan mugimenduan eta ilunpean. ABC metodoaz baliatuz injektatu genuen antigorputz biotinilatuaren seinalea areagotzen zen.

Hurrengo egunean, PBSan (0,1 M pH 7,4) 5 minutuko 2 garbiketa eta hirugarren bat Tris HCl-an egin genituen. Errebelatzeko diaminobenzidina (300 µl DAB eta 8,3 µl ur oxigenatu 50 ml Tris-HCl-an 0,05 M pH 7,4) gehitu eta 5 eta 10 minutu bitartean utzi genuen, erreakzioak kolorea hartu arte. Erreakzioa gelditzeko 5 minutuko garbiketa bat egin genuen Tris HCl-an (0,05 M pH 7,4) eta beste bi PBS 0,1M-an. Azkenik, laginak porta gelatinizatuetan jarri eta deshidratatu genituen graduazioan gorantz doan alkoholen laguntzaz, xilenotan 5 minututako 2 bainu egin eta ebakinak porta-estalkiarekin estali genituen DPX erretxina erabiliz.

BDA injekzioa jaso zuten burmuinetan konexio neuronalen azterketa egiteko, laginak mikroskopio bidez (Optiphot-2 Nikon) begiratu genituen 10 eta 40 biderreko handipenarekin. Era berean, zonalde desberdinetan argazkiak atera genituen Nikon Digital Camera DMX 1200F mikroargazkien sistema bat erabiliz.

4.12. AZTERKETA ESTATISTIKOA

Emaitzen analisi estatistikorako, parametro kuantitatiboen batez bestekoa eta horren errore estandarra (*Standar Error of the Mean*, SEM) kalkulatu ziren, eta taldeen arteko desberdintasunak faktore bakarreko bariantzaren analisi anitza (*ANalysis Of VAriance*, ANOVA) eta osteko Bonferroni-Dunn-ren zuzenketa erabiliz aztertu egin ziren. Aldaketak esangarriak bezala onartzeko $P<0.0001$ ala $P<0.05$ balioak aintzat hartu ziren kasuaren arabera. Analisi estatistiko guztietarako GraphPad Prism version 5.0 (Graph Pad Software, San Diego, CA, USA) programa erabili izan zen.

5. RESULTS

5.1. MORPHOLOGY STUDY

5.1.1. ANTIOXIDANT ADMINISTRATION AVOIDED THE LOSS OF BODY WEIGHT

At postnatal day 7 (P7), we compared the body weight of each animal and no differences were found among the experimental groups (Figure 18A). In contrast, 7 days after (P14), statistically significant differences were observed (P<0.0001). Animals that underwent hypoxic-ischemic injury presented less weight in comparison to control group. Animals pretreated with resveratrol and docosahexaenoic acid obtained similar values to control ones, but not the group that received the combination of both antioxidants (Figure 18B).

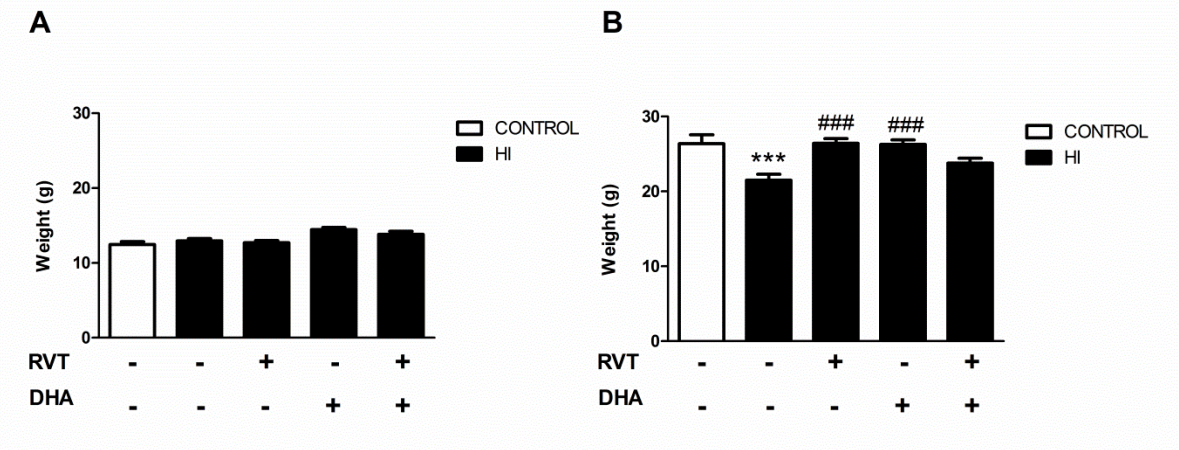


Figure 18: Body weight (expressed in grams) of P7 (A) and P14 (B) rats of the different experimental groups expressed as the mean ± SEM. Asterisks denote the significance levels when compared to the control group ($^{***}P$<0.0001). The hash symbols denote the significance levels when compared to the HI group ($^{###}P$<0.0001). It is showed how hypoxia-ischemia results in a significant lack of gain in body weight 7 days after the injury, while animals that received resveratrol and DHA obtained similar values to control ones, with the exception of the group that received the combination of both antioxidants.

The results from body weight suggest that hypoxia-ischemia decreases somatic growth, while antioxidant administration avoids this diminishment.

5.1.2. ANTIOXIDANT PRETREATMENT PROTECTED AGAINST BRAIN INJURY AND TISSUE LOSS

Nissl staining was performed to evaluate the possible damage on tissue 7 days after hypoxic-ischemic injury. Representative photographs of coronal sections of the perinatal brains of the different experimental groups are showed in Figure 19. Retraction of the ipsilateral hippocampus and loss of cortical volume as a consequence of the increased ventricular size were characteristics of the HI group in P14 animals. In general these brains revealed an infarct area in the ipsilateral side with loss of brain tissue (Figure 19B), while no macroscopic differences could be observed between control (Figure 19A) and treated animals (Figure 19 C, D, E).

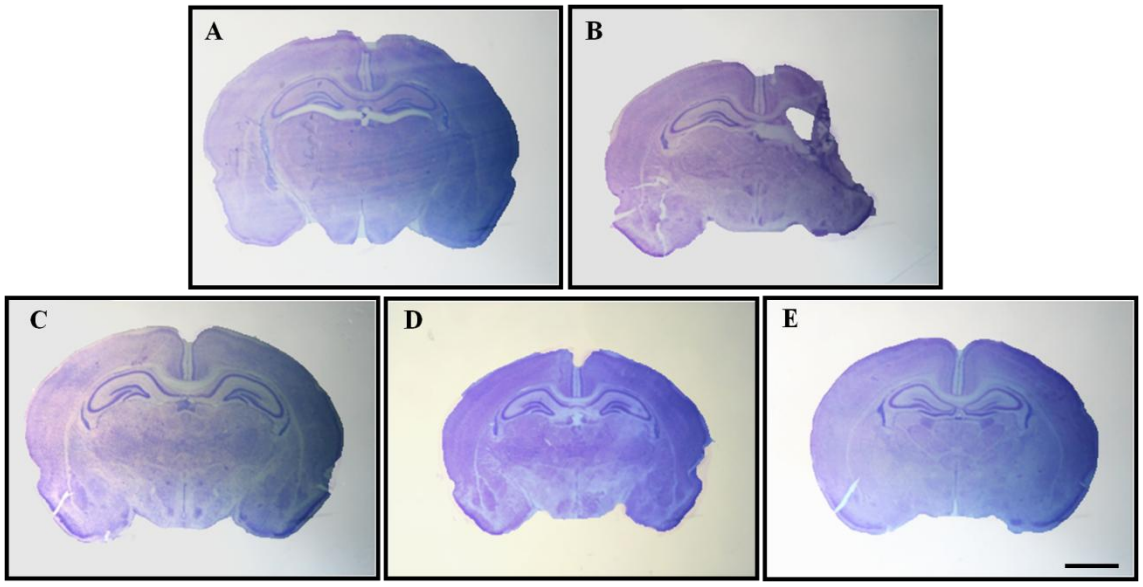


Figure 19: Brain tissue loss induced by hypoxia-ischemia in 7 day-old rats (P7) and evaluated at P14. Representative stereomicroscopic photographs of 5 µm Nissl-stained brain sections (interaural distance 5.40 mm and bregma -3.60 mm) are shown. (**A**) Control group with normal morphological brain. (**B**) HI brain with evident loss of tissue in the ipsilateral side of the cortex, and with obvious damage to the hippocampus. (**C**) HI brain treated with resveratrol, which is similar to the control brain. (**D**) HI Brain treated with docosahexaenoic acid, which is similar to the control brain. (**E**) HI brain treated with resveratrol and docosahexaenoic acid, which is similar to the control brain. Scale bar: 2.5 mm.

A quantitative evaluation of infarct area is shown in Figure 20. The area of infarction was defined as the loss of the normal cresyl violet staining pattern measured using Image J software. The HI group was found to have a high percentage of damage [30.1% (± 2.4)] in comparison to control [0.9% (± 1.2)], while no statistical difference (P<0.0001) was found between animals pretreated with antioxidants [RVT (5.1% ± 0.6); DHA (4.51±0.49); RVT+DHA (4.55±0.45)] and control group.

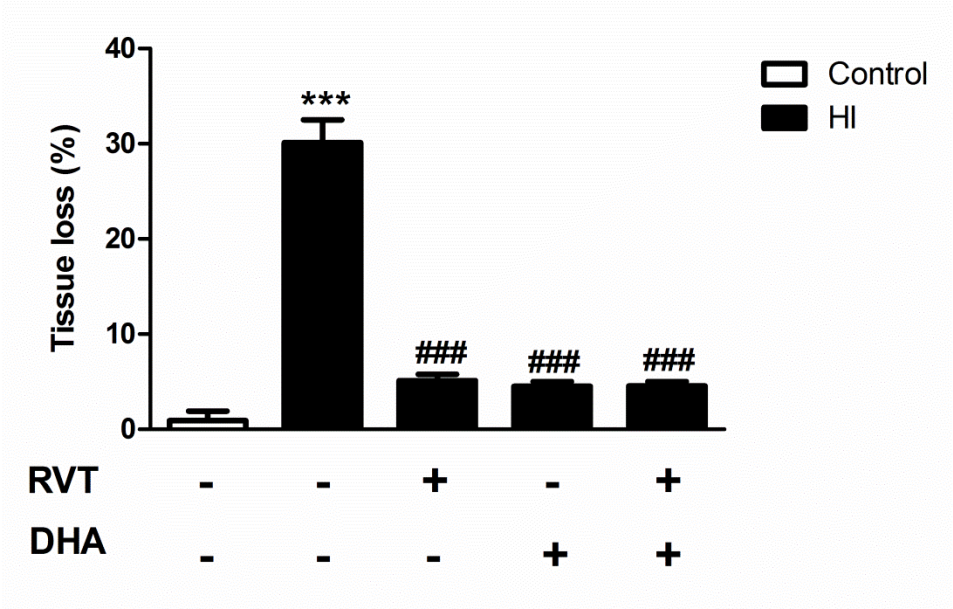


Figure 20: Quantification of the infarct area in 14 day-old newborn rats of the different experimental groups. The histogram shows the percentage of tissue loss, expressed as a mean ± SEM, which was calculated from the corresponding bihemispheric area ratio, using the formula ([C-I])/C*100 where C is the cresyl violet stained area of the contralateral hemisphere and I is this value for the ipsilateral hemisphere. Asterisks denote the significance levels when compared to the control group ($^{***}P$<0.0001). The hashes denote the significance levels when compared to the HI group ($^{###}P$<0.0001).

The photographs from the whole brain and the quantitative analysis demonstrate that the brains of the animals that underwent hypoxic-ischemic injury presented an infarct area in the ipsilateral side with loss of brain tissue. In contrast, no signs of infarct could be observed in animals pretreated with antioxidants.

5.1.3. ANTIOXIDANTS REDUCED CELL DAMAGE IN THE HIPPOCAMPUS AND IN THE PARIETAL CORTEX

The hippocampus and the parietal cortex of hypoxic-ischemic animals displayed significant evidence of infarction, whereas those of control animals did not (Figure 21) In contrast, animals pretreated with antioxidants did not show any infracted area.

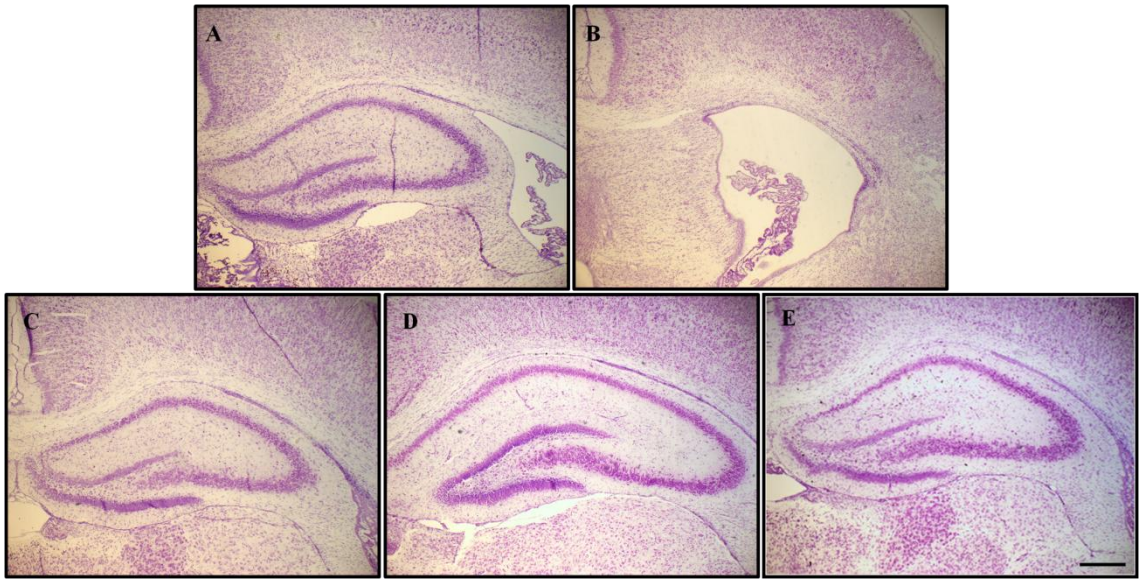


Figure 21: Representative microphotographs of Nissl-stained ipsilateral hippocampus in animals exposed to hypoxia-ischemia at P7 and evaluated at P14. Individual fields represent different experimental groups: (**A**) control, (**B**) HI, (**C**) RVT, (**D**) DHA and (**E**) RVT+DHA. HI group presented an evidential loss of tissue and retraction of hippocampus, whereas pretreated animals demonstrated similar morphological hippocampus respect to the control group. Scale bar: 80 µm.

The second way to quantify the damage caused by hypoxia-ischemia was by using a semi-quantitative histopathological scoring system, modified from the one presented by Hedtjärn et al. (2002). Results from this histopathological scoring (Figure 22 and 24) corroborate those seen in microscopic images (Figure 21 and 23). Thus, in whole hippocampus (the sum of each cell area) (P<0.0001) animals that received a single dose of antioxidants got significantly lower punctuation [RVT (5.71), DHA (3.15), RVT+DHA (4.25)] comparing with HI group (15.13) (Figure 22A).

The overall, total score was also significantly (P<0.0001) reduced in the pretreated groups [RVT (9.25), DHA (7.14), RVT+DHA (5.75)] versus the 22.25 points of the HI group (Figure 22B).

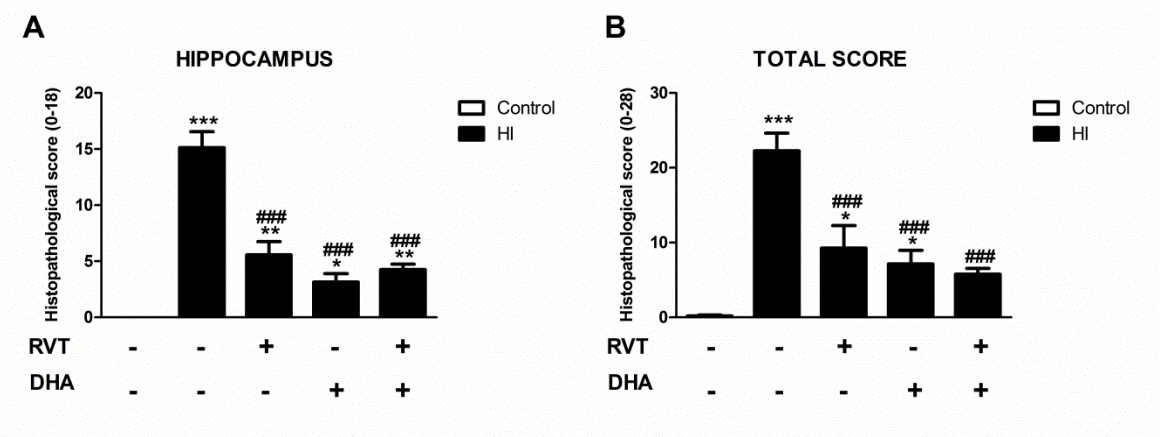


Figure 22: Histopathological score of damage in P14 rats brain. In (A) the hippocampus and in (B) the whole brain of different groups is expressed as the mean ± SEM. Asterisks denote the significance levels when compared to the control group (*****P<0.05, ******P<0.0005 or *******P<0.0001). The hash symbols denote the significance levels when compared to the hypoxia-ischemia group ($^{\#\#\#}P$<0.0001). It can be clearly seen that the groups pretreated with antioxidants (RVT, n=8; DHA, n=14; RVT+DHA, n=8) had a lower histopathological score compared with the HI group (n=8) in the whole brain (total score) and hippocampus, showing similar values to the control group (n=11).

Microscopic evaluation of brain damage in the different groups was made by analyzing the CA 1, CA 2-3 and dentate gyrus of the hippocampus and the parietal cortex, with a light microscope (x400). In Nissl stained brain sections, hypoxia-ischemia induced a significant cell loss (Figure 23). Moreover, asphyctic animals showed swollen and deformed neurons especially in the ipsilateral CA 1 and CTX areas. In contrast, only mild cell loss and a few damaged neurons were observed in slices from animals pretreated with antioxidants, in all of the brain regions studied. Overall, the subfields in hippocampus and the parietal cortex were similar in structure to those of the control group.

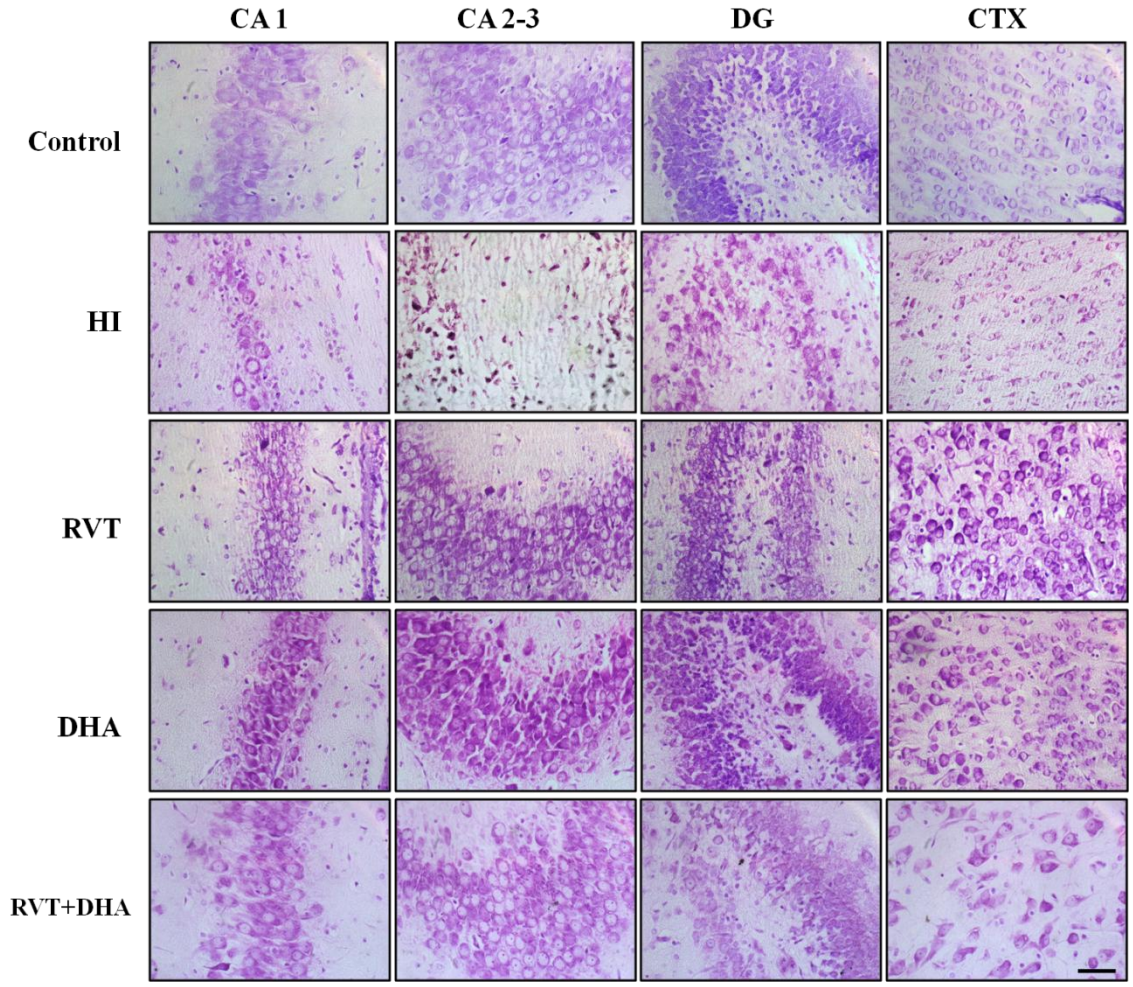


Figure 23: Representative microphotographs of Nissl-stained brain sections in animals exposed to hypoxia-ischemia at P7 and evaluated at P14. Individual fields represent different experimental groups and different areas of the hippocampus (CA 1, CA 2-3 and DG) and from the parietal cortex (CTX). Cell loss is especially evident in the CA-1 and parietal cortex in the hypoxia-ischemia. In contrast, animals pretreated with antioxidant treatments showed a remarkable conservation in the cellularity of the different studied areas with respect to the HI group. Scale bar: 50 μm.

In the same way, animals that received resveratrol, DHA and the combination of both before hypoxia demonstrated significantly lower values of neuropathology in CA 1, CA 2-3, DG and CTX, than those of the HI group ($P<0.0001$). This improvement was more evident in the dentate gyrus and parietal cortex, with values being similar to those of controls (Figure 24).

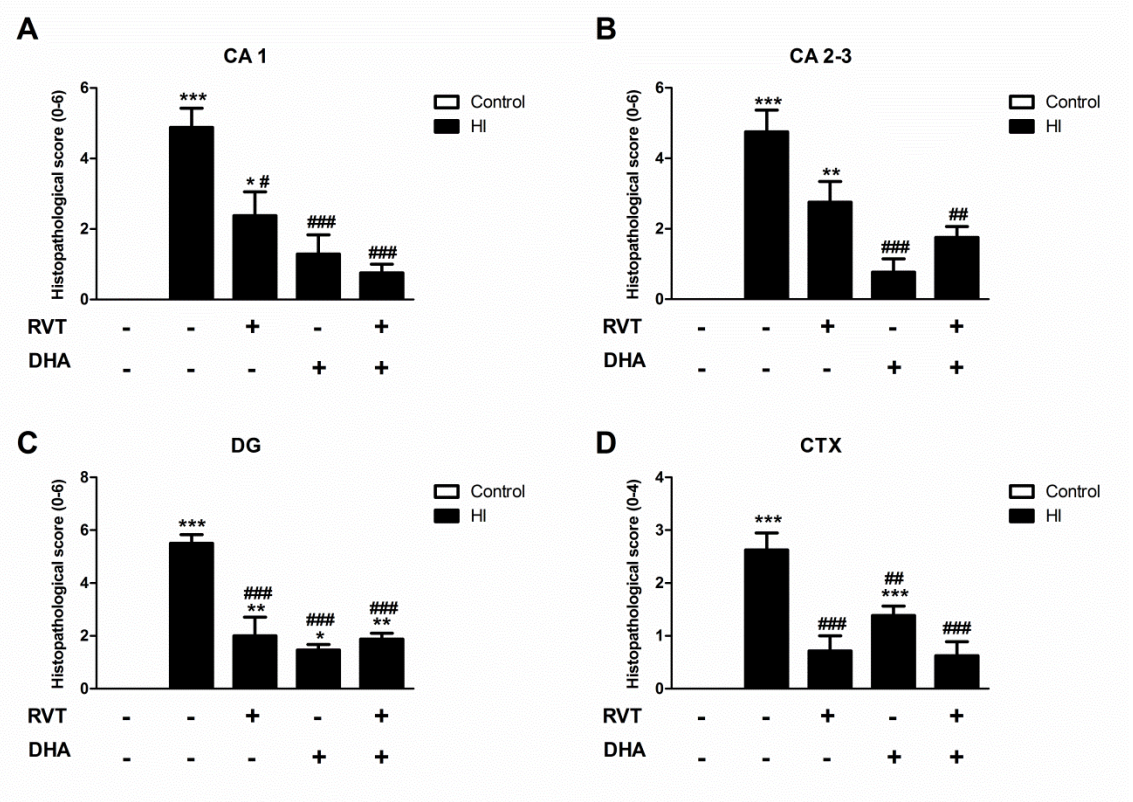


Figure 24: Histopathological score of damage in P14 rats brain in (**A) CA 1, (B) CA 2-3, (C) DG areas of the hippocampus and (D) CTX** of different groups expressed as the mean ± SEM. Asterisks denote the significance levels when compared to the control group (******P*<0.05, *******P*<0.0005 or ********P*<0.0001). The hash symbols denote the significance levels when compared to the hypoxia-ischemia group ($^{\#}P$<0.05, $^{\#\#}P$<0.0005 or $^{\#\#\#}P$<0.0001). It can be clearly seen that the groups pretreated with antioxidants (RVT, n=8; DHA, n=14; RVT+DHA, n=8) had a lower histopathological score compared with the HI group (n=8) in all the regions analyzed in the hippocampus and the parietal cortex, showing similar values to control group (n=11).

Antioxidants reduced cell damage in the hippocampus and in the parietal cortex, avoiding the cell loss and the damage to cells seven days after the injury, showed a remarkable conservation of cellularity.

5.1.4. BOTH RESVERATROL AND DOCOSAHEXAENOIC ACID AND THEIR COMBINATION REDUCED GLIAL FIBRILARY ACID PROTEIN IMMUNOREACTIVITY

High levels of GFAP astrogliosis were found in animals with hypoxic-ischemic injury, particularly in regions near dead or dying cells, such as the CA1 and dentate gyrus areas of the hippocampus, whereas control cases showed low levels of GFAP immunoreactivity (Figures 25 and 26). This reactive astrocyte response was diminished when antioxidant treatments were administered.

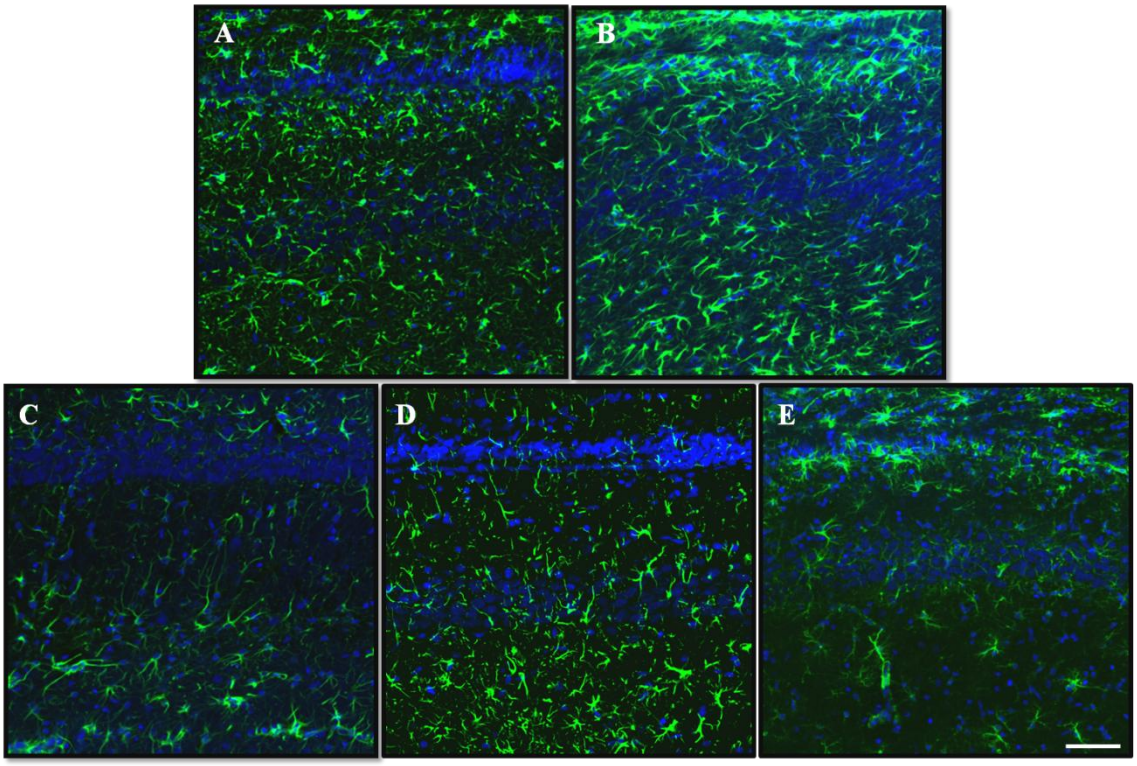


Figure 25: Representative confocal microphotographs of glial fibrillary acidic protein (GFAP)-stained brain sections counterstained with DAPI, in the CA 1 area of the hippocampus, of the five experimental groups (n=8): (**A**) control, (**B**) HI, (**C**) RVT, (**D**) DHA and (**E**) RVT+DHA. 7 days after the hypoxic-ischemic injury, HI groups showed an increase in the GFAP immunoreactivity (green) in this area, comparing with the control group, whereas this reactivity is substantially reduced in animals pre-treated with antioxidants. Scale bar: 40 µm.

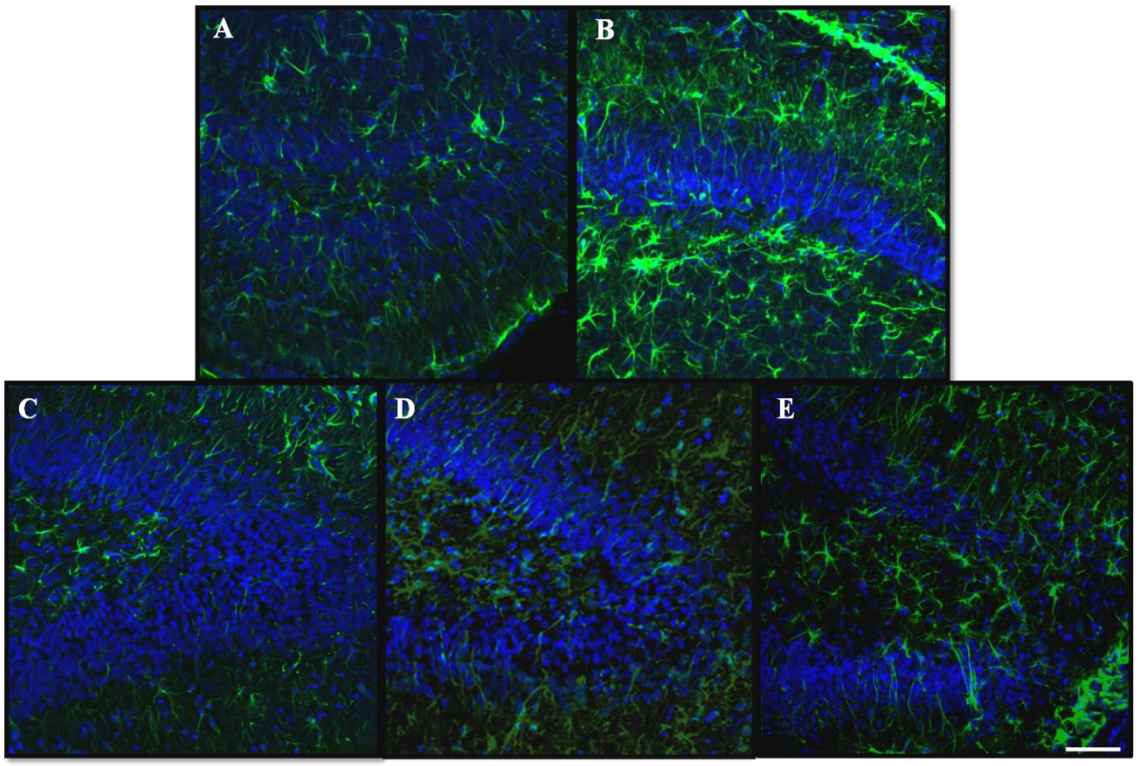


Figure 26: Representative confocal microphotographs of GFAP-stained brain sections counterstained with DAPI, in the dentate gyrus area of the hippocampus, of the five experimental groups (n=8): (**A**) control, (**B**) HI, (**C**) RVT, (**D**) DHA and (**E**) RVT+DHA. On P14, GFAP immunoreactivity was substantially reduced in animals pre-treated with antioxidants comparing with HI animals. Scale bar: 40 μm.

The GFAP-immunoreactivity was diminished when antioxidant treatments were administered, particularly in the CA 1 and dentate gyrus areas of the hippocampus.

5.1.5. THE LOSS OF MBP IMMUNOSTAINING WAS ABSENT WITH ANTIOXIDANT THERAPIES

MBP immunostaining pattern showed differences among groups at the level of the external capsule (Figure 27) and mid-striatum (Figure 28), evaluated at P14. A substantial loss of ipsilateral MBP immunostaining was observed in both areas of animals that underwent hypoxia-ischemia respect to controls, while this loss was absent with all the antioxidant therapies.

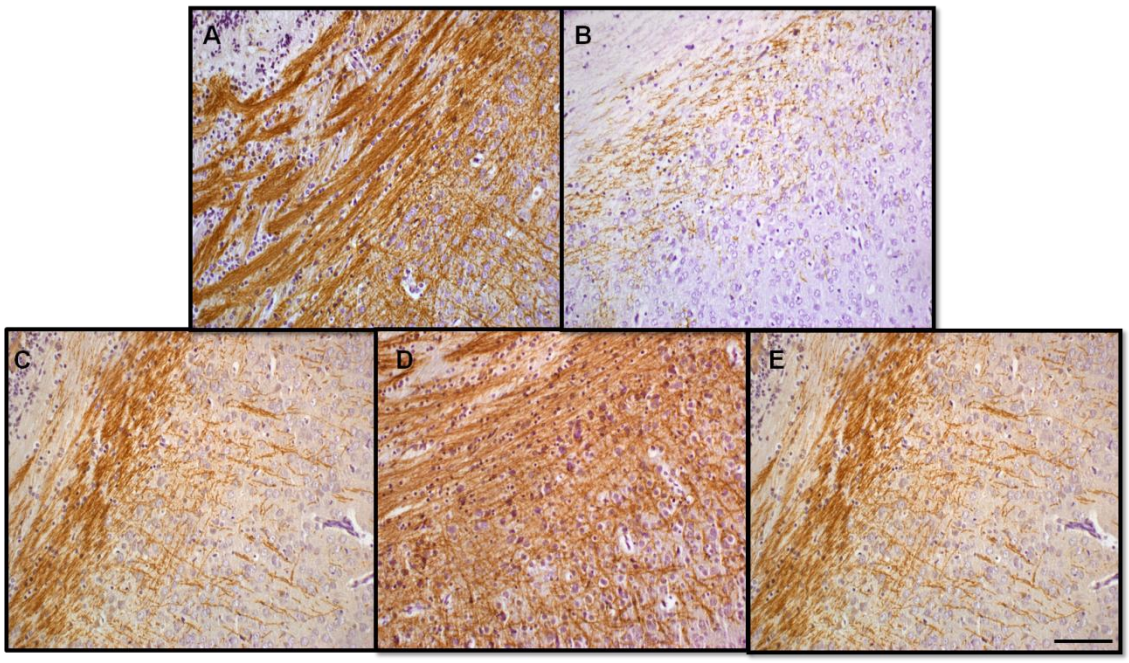


Figure 27: Representative light microphotographs of myelin basic protein (MBP)-stained brain sections in the external capsule of the five experimental groups (n=8): (**A**) control, (**B**) HI, (**C**) RVT, (**D**) DHA and (**E**) RVT+DHA. Scale bar: 40 µm.

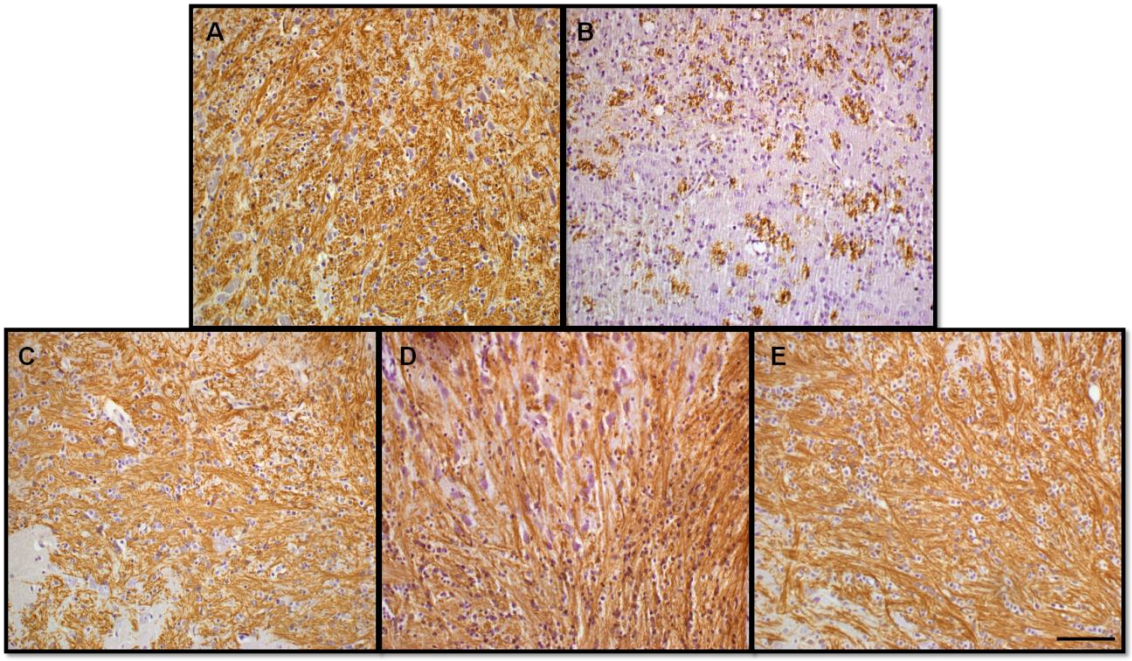


Figure 28: Representative light microphotographs of myelin basic protein (MBP)-stained brain sections of the mid-striatum of the five experimental groups (n=8): (**A**) control, (**B**) HI, (**C**) RVT, (**D**) DHA and (**E**) RVT+DHA. Scale bar: 40 µm.

5.1.6. ANTIOXIDANT PRETREATMENT PRESERVED MYELINATION

White matter integrity was analyzed by measuring the density of MBP immunostaining of both areas of the brain, so densitometric values were expressed as ratios of ipsilateral-to-contralateral hemispheric measurements (I:C).

Quantitative analysis corroborated what was apparent at a microscopical level. At the level of the external capsule (Figure 29), a substantial loss of ipsilateral MBP immunostaining (P<0.0005) was measured in the HI group (0.86) when compared with control animals (0.99). Pups pretreated with antioxidants showed a smaller degree of MBP loss in the ipsilateral hemisphere [RVT (0.98), DHA (0.99), RVT+DHA (0.95)].

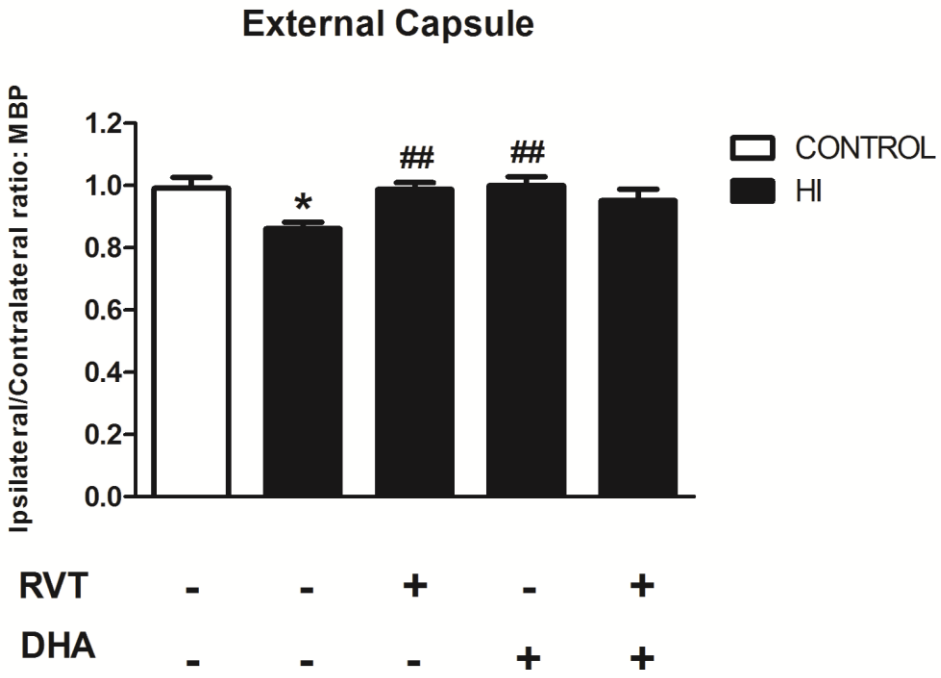


Figure 29: Comparison of loss of MBP immunostaining in the external capsule of the five experimental groups: (A) Control (n=5), (B) HI (n=14), (C) RVT (n=14), (D) DHA (n=11) and (E) RVT+DHA (n=6). In the histogram, the extent of tissue injury, expressed as a ratio of left-to-right hemispheric MBP immunostaining is represented. Asterisks denote the significance levels when compared to the control group (*****P<0.05). Hashes denote the significance level when compared to the HI group ($^{\#\#}P$<0.0005).

At the level of the mid-striatum (Figure 30), HI neonatal rats (0.66) exhibited a significant (P<0.0001]) loss in MBP immunostaining in subcortical white matter when compared with control animals (1.01), whereas pups receiving a single dose of treatment obtained similar (I:C) MBP ratio to that observed in control [RVT (1.01), DHA (1.01), RVT+DHA (1.01].

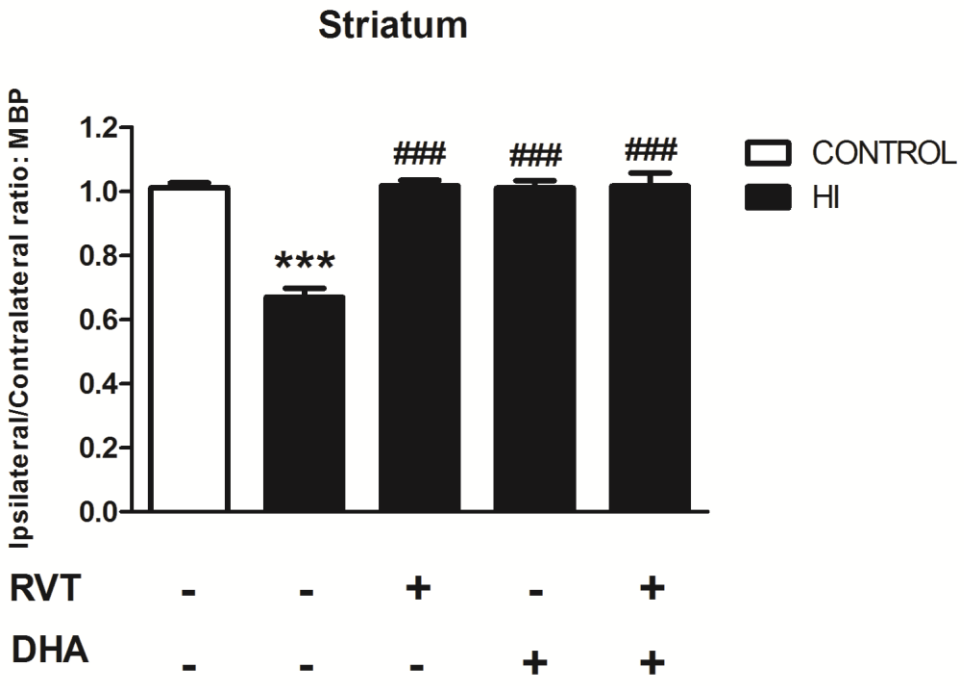


Figure 30: Comparison of loss of MBP immunostaining in the mid-striatum of the five experimental groups: (A) Control (n=6), (B) HI (n=14), (C) RVT (n=12), (D) DHA (n=11) and (E) RVT+DHA (n=6). In the histogram, the extent of tissue injury, expressed as a ratio of left-to-right hemispheric MBP immunostaining is represented. Asterisks denote the significance levels when compared to the control group (**P<0.0005). Hashes denote the significance level when compared to the HI group ($^{###}P$<0.0001).

These results indicate that resveratrol and docosahexaenoic acid both individually and the combination of both are able to ameliorate the loss of myelination in the external capsule and the striatum at the level of the middle hippocampus.

5.1.7. ANTIOXIDANTS REDUCED MICROGLIAL ACTIVATION

IBA-1 immunostaining pattern showed differences among groups in the CA 1 (Figure 31) and dentate gyrus (Figure 32) areas of the hippocampus, evaluated 7 days after the injury. A substantial increase of ipsilateral Iba-1 immunostaining was observed in both areas of animals that underwent hypoxia-ischemia respect to controls, while this increase was no so evident with all the antioxidant therapies.

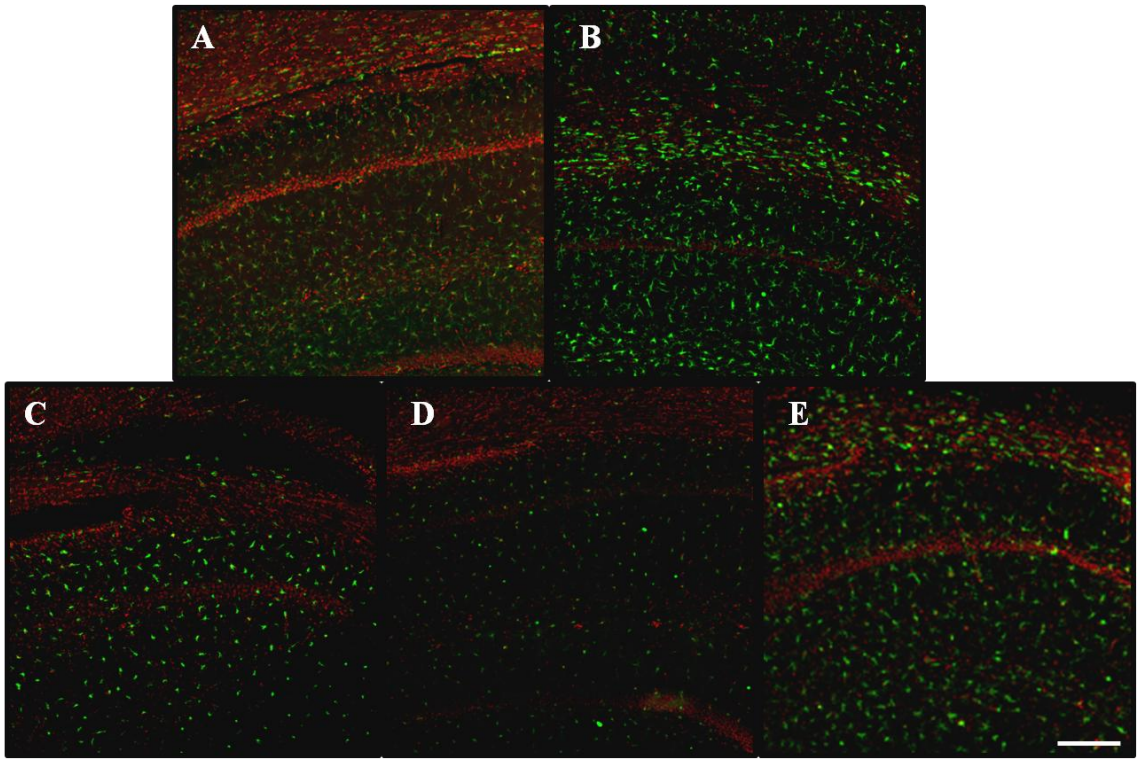


Figure 31: Representative confocal microphotographs of IBA-1-stained brain sections counterstained with DAPI (red), in the dentate CA 1 area of the hippocampus, of the five experimental groups (n=8): (**A**) control, (**B**) HI, (**C**) RVT, (**D**) DHA and (**E**) RVT+DHA. On postnatal day 14, IBA-1 (green) immunoreactivity was substantially reduced in animals pre-treated with antioxidants comparing with HI animals. Scale bar: 40 μm.

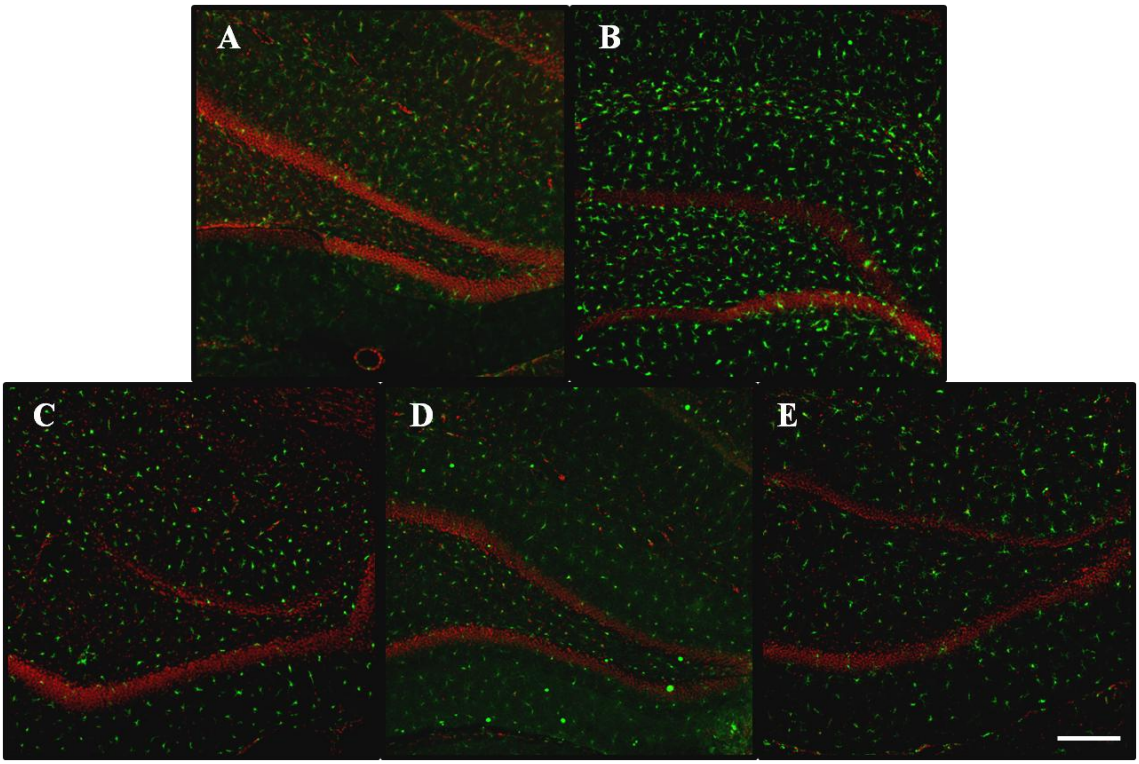


Figure 31: Representative confocal microphotographs of IBA-1-stained brain sections counterstained with DAPI (red), in the dentate dentate gyrus area of the hippocampus, of the five experimental groups (n=8): (**A**) control, (**B**) HI, (**C**) RVT, (**D**) DHA and (**E**) RVT+DHA. 7 days after the hypoxic-ischemic injury, HI groups showed an increase in the IBA-1 immunoreactivity (green) in this area, comparing with the control group, whereas this reactivity is substantially reduced in animals pre-treated with antioxidants. Scale bar: 40 μm.

These data suggest that antioxidants reduce microglial activation in CA1 and dentate gyrus areas of the hippocampus.

5.2. ASSESSMENT OF MITOCHONDRIAL STATE

We analyzed the mitochondrial state by focusing on its integrity and functionality and its production of oxygen reactive species, as all of these are altered parameters after the metabolic cascade triggered by hypoxic-ischemic injury.

Those parameters were evaluated in suspension of acutely isolated cells from ipsilateral side of rats brains, by using different fluorochromes in the different experimental groups at three time points of study, 0 h, 3 h and 12 h after hypoxic-ischemic event. The results obtained using flow cytometer are expressed as mean ± SEM, for the percentage of cell positive to those fluorochromes and for the fluorescence intensity relative to control group.

5.2.1. MITOCHONDRIAL INNER MEMBRANE INTEGRITY WAS PROTECTED BY ANTIOXIDANTS

The level of cardiolipin was determined by using the fluorochrome nonyl acridine orange (NAO). This marker binds to cardiolipin that is located in the mitochondrial internal membrane and is essential for protein functionality and ATP synthesis.

At first studied state, at 0 h, there were no statistically significant differences in the percentage of NAO positive cells among all groups (Figure 32A).

At 3 h, there were no differences between control and HI, but RVT group presented a significant reduction, but not the rest of the treated groups (Figure 32B).

On the contrary, at 12 h (P<0.05), HI group underwent a diminishment with statistical differences (76.53±10.71) with respect to the control group in the percentage of positive cells (97.37±0.61). Meanwhile animals pretreated with antioxidants maintained mitochondrial inner membrane integrity [RVT (99.42±0.06); DHA (98.29±0.84); RVT+DHA (98.83±0.39)] and showed similar values in comparison to the control group (Figure 32C).

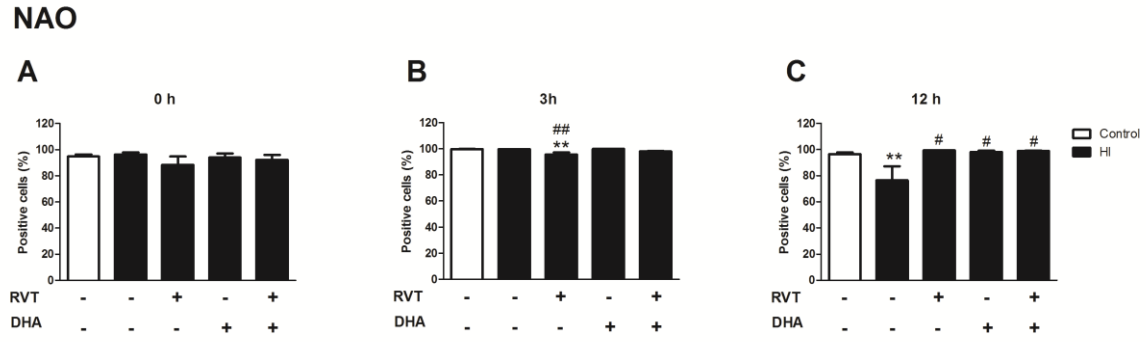


Figure 32: Mitochondrial inner membrane integrity evaluation in suspension of acutely isolated cells using nonyl acridine orange (NAO). The percentage of positive cells with in vivo marker NAO at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in the different experimental groups, control (n≥5), HI n≥5), RVT (n≥5), DHA (n≥5) and RVT+DHA (n≥5). Asterisk denotes the significance levels when compared to the control group (*****P<0.05 and *****P<0.005). The hash symbol denotes the significance levels when compared to the HI group ($^{\#}P$<0.05 and $^{\#\#}P$<0.005).

Regarding fluorescence intensity, at first studied state, at 0 h (P<0.005), HI group underwent a diminishment but without statistical differences in the relative values of fluorescence intensity for NAO with respect to the control group. Pretreated groups showed similar values to control group, although RVT+DHA presented a significant augmentation (Figure 33A).

At 3 h there was no significant difference among the groups (Figure 33B), apart from the RVT+DHA group, which suffered a significant diminishment (P<0.05).

At 12 h (P<0.05) HI group underwent a diminishment but without statistical differences in the relative values of fluorescence intensity for NAO with respect to the control group. After antioxidant administration the relative values of fluorescence intensity for NAO were significantly higher than HI group and even control, especially in the group treated with DHA (Figure 33C).

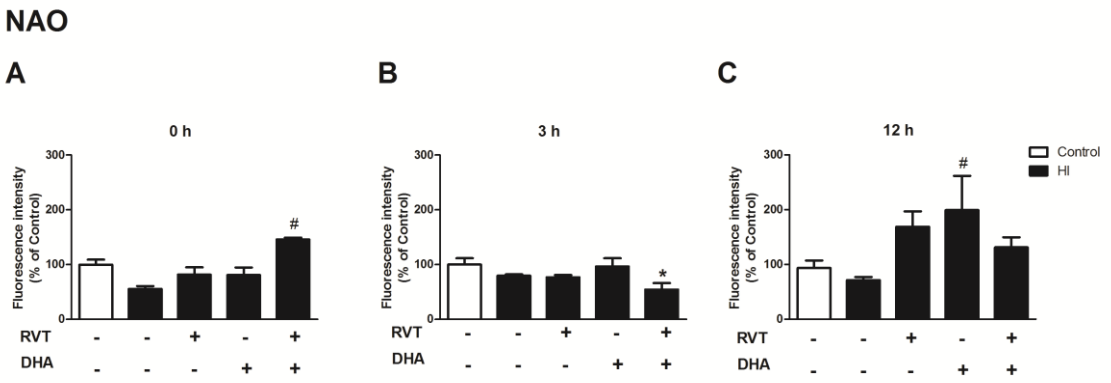


Figure 33: Mitochondrial inner membrane integrity evaluation in suspension of acutely isolated cells using nonyl acridine orange (NAO). The relative fluorescence intensity of cells with in vivo marker NAO at different points of time after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, control (n≥5), HI (n≥5), RVT (n≥5), DHA (n≥5) and RVT+DHA (n≥5). Asterisk denotes the significance levels when compared to the control group (*****P<0.05). The hash symbol denotes the significance levels when compared to the HI group ($^{\#}P$<0.05).

The data obtained from both the percentage of positive cells with NAO and the relative fluorescence intensity in acutely isolated cells suggest that antioxidants protected mitochondrial inner membrane integrity.

5.2.2. ANTIOXIDANTS MAINTAINED MITOCHONDRIAL TRANSMEMBRANE POTENTIAL

Mitochondrial transmembrane potential was analyzed by Rhodamine 123 (Rh 123), a lipophilic cationic fluorochrome, which is inside the mitochondria in a proportional way to the intensity of its mitochondrial transmembrane potential.

At 0 h (P < 0.0005), hypoxia-ischemia generated a decrease of the percentage of Rh 123 positive cell (82.89±3.78), in comparison to the control group (98.14±0.47). In contrast, animals pretreated with antioxidants showed a percentage of Rh 123 positive cells [RVT (93.25±1.18); DHA (95.95±1.95); RVT+DHA (95.95±0.68)] similar to the control group (Figure 34A).

At 3 h ($P < 0.0005$) there was a diminishment in cells isolated from HI rats (91.99±2.28), statistically not so significant, and also in the resveratrol pretreated group (92.91±1.71), respect to the control (99.88±0.03). In contrast, rats pretreated with DHA (99.92±0.06) and with RVT+DHA (95.9±1.2) showed similar values to the control group (Figure 34B).

However, at 12 h ($P < 0.0001$) animals subjected to the hypoxic-ischemic event underwent again an important diminishment in the percentage of cells with intact mitochondrial transmembrane potential (76.08±3.91), in comparison to the control group in the percentage of positive cells (98.81±0.37), while cells isolated from HI animals pretreated with antioxidants showed similar values [RVT (90.39±1.59); DHA (93.92±2.2); RVT+DHA (90.65±4.98)] to those of the control (Figure 34C).

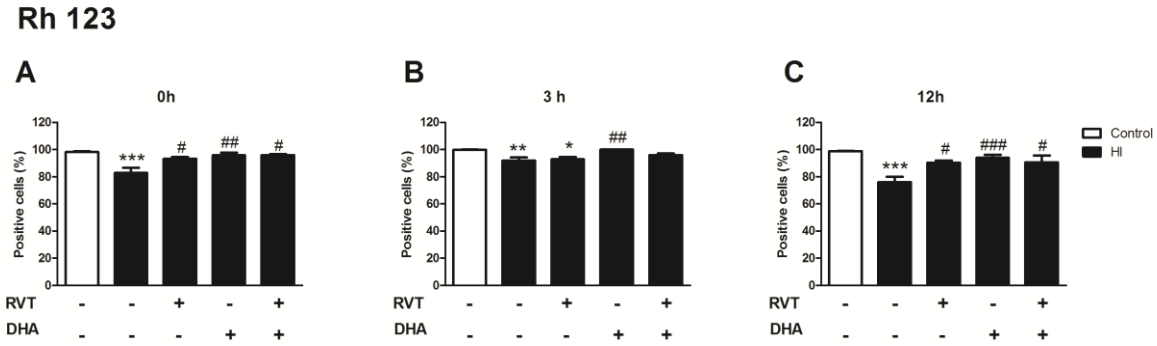


Figure 34: Mitochondrial transmembrane potential in suspension of acutely isolated cells measured as the percentage of positive cells with in vivo marker Rh 123 at different time points after hypoxia-ischemia, (**A**) **0 h, (B) 3 h and (C) 12 h**, in control (n≥5), HI (n≥5) and animals pretreated with antioxidants (n≥5). Asterisks denote the significance levels when compared to the control group (*****P<0.05 or *******P<0.0001). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05 or $^{\#\#\#}P$<0.0001).

Concerning the relative values of fluorescence intensity of Rh 123, at 0 h (P<0.0005), HI group underwent a diminishment but without statistical differences, so there was no significant difference among the groups, except for the RVT+DHA group which showed significant higher relatives values of fluorescence intensity of Rh 123 than cells isolated from HI rats and even higher than the control group (Figure 35A).

At 3 h (P<0.05) animals that received resveratrol and DHA obtained higher values than HI group (Figure 33B). At 12 h there was no significant difference among the groups (Figure 35C).

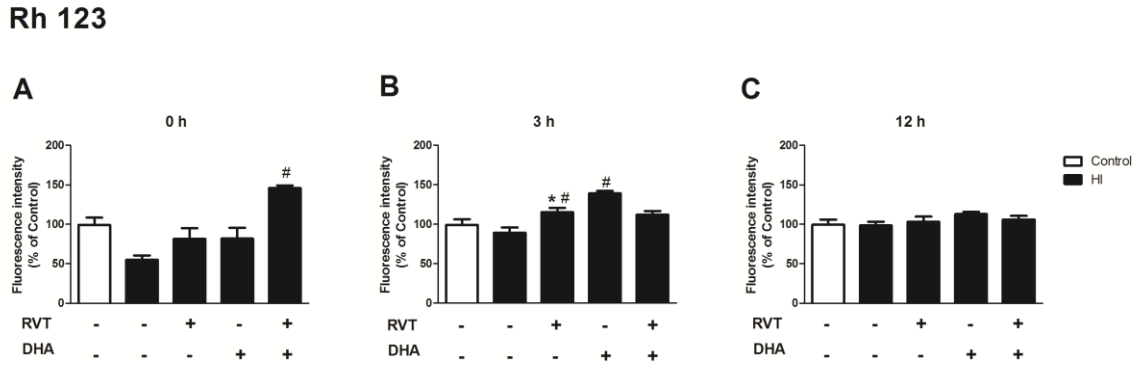


Figure 35: Mitochondrial transmembrane potential in suspension of acutely isolated cells using measured as the relative fluorescence intensity of cells with in vivo marker Rh 123 at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control (n≥5), HI (n≥5) and animals pretreated with antioxidants (n≥5). Asterisk denotes the significance levels when compared to the control group (*****P<0.05). The hash symbol denotes the significance levels when compared to the HI group ($^{\#}P$<0.05).

The results obtained from both the percentage of positive cells and the relative fluorescence intensity of Rh 123 from suspension of acutely isolated cells showed that antioxidants maintained the mitochondrial transmembrane potential. A decrease in the number of cells and in the fluorescence of Rh 123 induced by neonatal hypoxia-ischemia indicates a loss of mitochondrial transmembrane potential.

5.2.3. ANTIOXIDANTS REDUCED REACTIVE OXYGEN SPECIES PRODUCTION

Intracellular reactive oxygen species were detected using fluorocrome 2′,7′-dichlorofluorescein diacetate (DCFH-DA). This probe is cell-permeable and is hydrolyzed intracellularly to the DCFH carboxylate anion that is retained in the cell. Two-electron oxidation of DCFH results in the formation of a fluorescent product, dichlorofluorescein (DCF), which can be monitored by flow cytometry.

At 0 h (P<0.0001) while HI group showed a decrease in the percentage of DCFH-DA positive cells (50.66±1.48), treated groups presented similar percentage values without statistical differences values [RVT (92.25±1.98); DHA (88.13±3.20); RVT+DHA (91.17±0.58)] with respect to the control group (90.45±1.55) (Figure 36A).

At 3 h there were not statistically significant differences between control and HI groups, but DHA and RVT+DHA animals showed higher values (P<0.05) that HI ones (Figures 36B). At 12 h there was no significant difference among the groups, apart from the DHA group, which presented a significant augmentation in the percentage of DCFH-DA positive cells comparing to HI group (P<0.005) (Figures 36C).

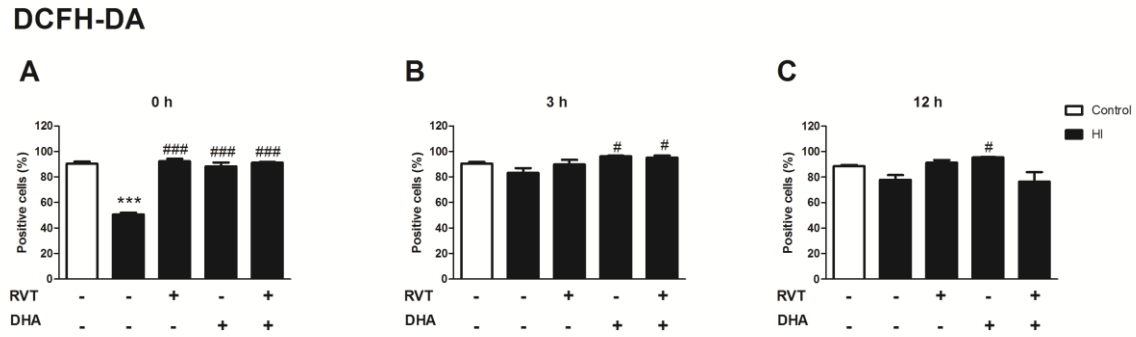


Figure 36: Production of reactive oxygen species in suspension of acutely isolated cells using measured as the percentage of positive cells with in vivo marker DCFH-DA at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control (n≥5), HI (n≥5) and animals pretreated with antioxidants (n≥5). Asterisks denote the significance levels when compared to the control group ($^{***}P$<0.0001). The hash symbols denote the significance levels when compared to the hypoxia-ischemia group ($^{\#\#\#}P$<0.0001).

Concerning the relative values of fluorescence intensity of ROS production, at 0 h there was no significant difference between HI and Control groups, but the DHA group suffered a significant diminishment (P<0.0005) comparing to control group (Figure 37A).

In contrast, at 3 h (P<0.0001) there was an increase in the cells isolated from HI rats (197.19±14.93) and also in RVT+DHA animals (236.6±4.26) (Figure 37B) comparing with the control group (100.2±8.58), but not in the rest of the treated groups

[RVT (159.4±15.64); DHA (128.2±22.73)]. At 12 h ($P < 0.0001$) the relative values of fluorescence intensity of ROS were significantly increased in HI (303.7±8.58), RVT (240.422±28.73) and DHA (316±15.97) groups respect to control one (96.6±7.39), but in case of the animals pretreated with a combination of resveratrol and docosahexaenoic acid showed similar values to control rats (103.9±22.9) (Figure 37C).

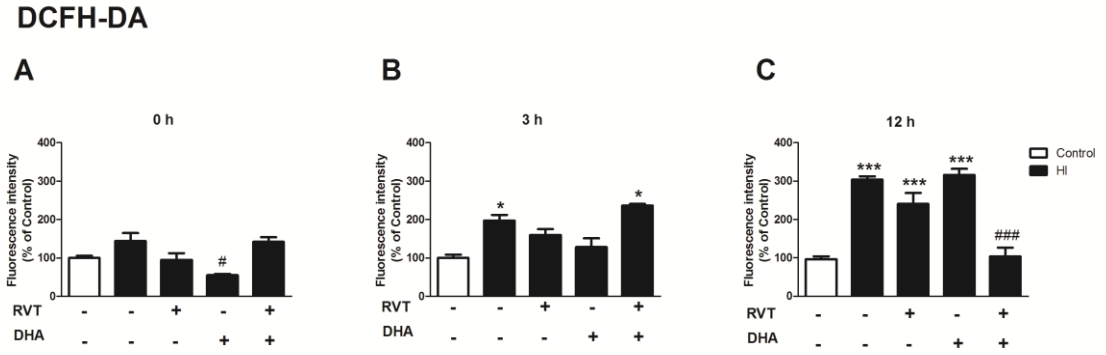


Figure 37: Production of reactive oxygen species in suspension of acutely isolated cells using measured as the relative fluorescence intensity of cells with in vivo marker DCFH-DA at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control (n≥5), HI (n≥5) and animals pretreated with antioxidants (n≥5). Asterisk denotes the significance levels when compared to the control group (*****P<0.05 and *******P<0.0001). The hash symbol denotes the significance levels when compared to the HI group ($^{\#}P$<0.05).

In the case of the production of reactive oxygen radicals, only the data of the relative fluorescence intensity showed that the combination of both antioxidants reduced the quantity in the cells that are producing them.

5.3. GENE EXPRESSION BY RT-qPCR

We quantified the expression of 20 genes in the ipsilateral side of the brain by quantitative real time PCR immediately after (0 h), 3 h and 12 h after the hypoxic-ischemic brain injury in all the experimental groups. The genes were grouped in five blocks: immediate early genes, implicated elements, oxidative stress, death pathways and inflammatory pathways.

We chose different reference genes for each time point using Genorm and Normfinder programs, which are algorithm for identifying the optimal normalization gene among a set of candidates: at 0 h, *Hprt* and *Pgk1*; at 3 h *B2M*, *Hprt* and *Ppia*; and at 12 h *Pgk1*, *Ppia* and *Rplpo*.

5.3.1. IMMEDIATE EARLY GENES

We aimed to investigate if the expression of immediate early genes was changed after hypoxic-ischemic brain injury in the different experimental groups. The immediate early genes that we evaluated were the hypoxia-inducible factor-1α (HIF-1α), heat shock factor 1 (H*sf1*), early growth response 1 (*Erg1*), FBJ osteosarcoma oncogen (*c-Fos*) and vascular endothelial growth factor (*Vegf*).

5.3.1.1. HYPOXIA-INDUCIBLE FACTOR-1α (*Hif1α*)

Following hypoxia-ischemia, *Hif1α* was down-regulated in the DHA group (fold-change -0.44; $P<0.05$), but there was no difference among the rest of the experimental groups (Figure 38A).

At 3 h, the RVT+DHA groups suffered an important diminishment in the expression of this gene (fold-change -0.64; $P<0.05$), but no significant difference was found among the rest of the groups (Figure 38B).

In the same way, at 12 h, there were no difference among the groups, except from RVT+DHA (fold-change -0.31; $P<0.05$) that was down-regulated comparing with HI group (Figure 38C).

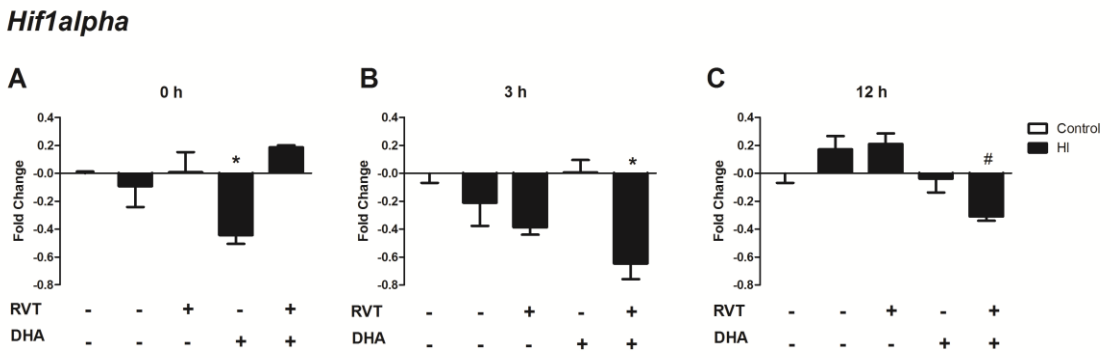


Figure 38: RT-PCR analysis of hypoxia-inducible factor 1α gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*P<0.05). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05).

5.3.1.2. HEAT SHOCK FACTOR 1 (*Hsf1*)

Immediately after the hypoxia-ischemia, *Hsf1* was significantly down-regulated in the HI group (fold-change -1.19; P<0.05) comparing with control. The animals that received any of the treatments suffered a diminishment but it was not statistically significant (Figure 39A).

3 h after the injury, the expression of heat shock factor 1 mRNA was increased in the DHA group (fold-change 0.7; P<0.05), comparing with both control and HI groups (Figure 39B).

At 12 h, HSF-1 was up-regulated in the ipsilateral side of the brain from both resveratrol (fold-change 0.54; P<0.05) and DHA (fold-change 0.48; P<0.05) animals, comparing with HI group (Figure 39C).

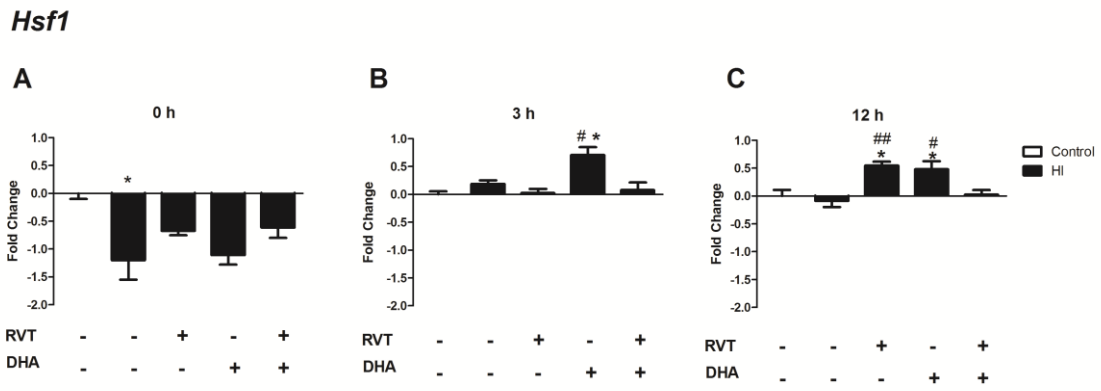


Figure 39: RT-PCR analysis of heat shock factor 1 gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (**P*<0.05). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05 or ($^{\#\#}P$<0.0005).

5.3.1.3. EARLY GROWTH RESPONSE 1 (*Egr1*)

Following hypoxia-ischemia, *Erg1* was up-regulated at in all the groups that underwent hypoxia-ischemia [fold-change: HI (1.21), RVT (1.93), DHA (1.45), RVT+DHA (1.2); *P*<0.05] respect to the control group (Figure 40A).

In the same way, at 3 h *Egr1* was also up-regulated in HI (fold-change 1.83; *P*<0.05), RVT (fold-change 2.34; *P*<0.05) and RVT+DHA (fold-change 1.75; *P*<0.05) groups comparing with control group. However, in the DHA group *Erg1* was down-regulated comparing with HI group (Figure 40B).

At 12 h, the pretreated groups suffer an augmentation of the expression of this gene [fold-change: RVT (0.71), DHA (0.52), RVT+DHA (0.79); *P*<0.05] respect the HI group (Figure 40C).

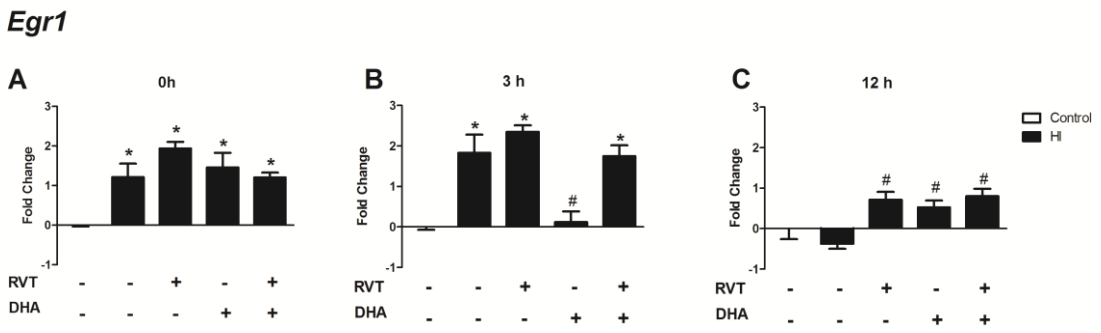


Figure 40: RT-PCR analysis of early growth response 1 gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*P<0.05). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05).

5.3.1.4. FBJ OSTEOSARCOMA ONCOGEN (*c-Fos*)

Immediately after the hypoxia-ischemia, *c-Fos* was significantly up-regulated in the HI group (fold-change 3.02; P<0.05) and animals pretreated with antioxidants [fold-change: RVT (4.42), DHA (4.11), RVT+DHA (2.49); P<0.05] comparing with control (Figure 41A).

This up-regulation was maintained 3 hours after the insult in the HI (fold-change 3.6; P<0.05), RVT (fold-change 4.76; P<0.05) and RVT+DHA (fold-change 4.08; P<0.05) groups in comparison to control, but not in the DHA group (Figure 41B).

In contrast, 12 hours the injury, there was no difference between HI and control groups, while animals that received any of the treatments suffered a significant up-regulation [fold-change: RVT (2.83), DHA (1.71), RVT+DHA (2.47); P<0.05], with respect to both control and HI groups (Figure 41C).

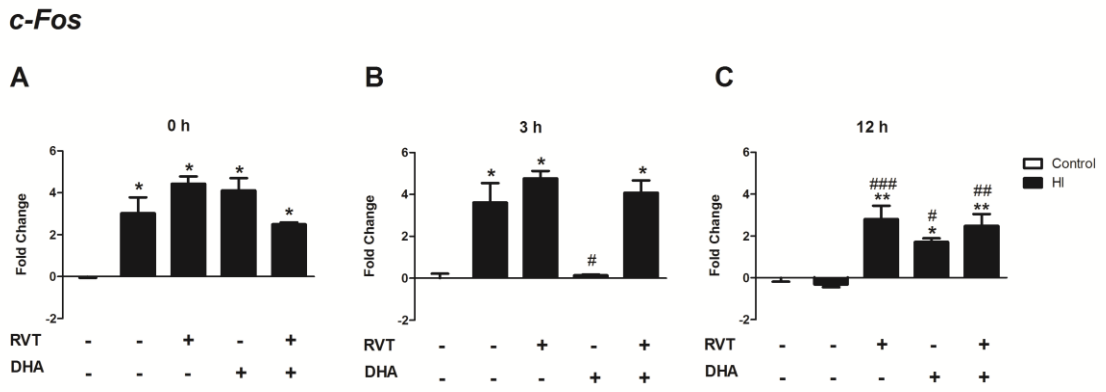


Figure 41: RT-PCR analysis of FBJ osteosarcoma oncogen gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*P<0.05 or **P<0.05). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05, $^{\#\#}P$<0.0005 or $^{\#\#\#}P$<0.0005).

5.3.1.5. VASCULAR ENDOTHELIAL GROWTH FACTOR (*Vegf*)

Following hypoxia-ischemia, *Vegf* was down-regulated in the HI group (fold-change -0.88; P<0.05), and in the RVT+DHA group it was up-regulated comparing with the HI group, showing similar values to control (Figure 42A).

In contrast, at 3 h, *Vegf* was up-regulated in all the animals that suffered hypoxic-ischemic injury [fold-change: RVT (0.68), DHA (0.71), RVT+DHA (0.69); P<0.05], but especially in the group that did not receive any treatment, the HI group (fold-change 1.06; P<0.0001) (Figure 42B).

On the contrary, the *Vegf* mRNA increased significantly in the HI group (fold-change: -0.52; P<0.05) at 12 h, but there was no difference among the rest of the groups (Figure 42C).

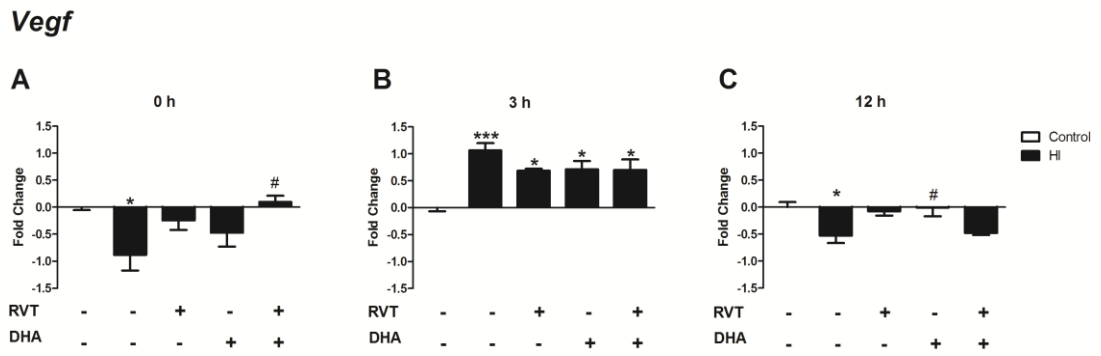


Figure 42: RT-PCR analysis of vascular endotelial growth factor gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*P<0.05 or ***P<0.0001). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05).

Regarding immediate early genes, *c-Fos* and *Erg1* were up-regulated in the HI group at 0 h and 3 h, but at 12 h no difference was observed between control and HI group. Animals pretreated with antioxidants presented an important increased in the expression of *c-Fos* and *Erg1* in all the time points, except in the case of DHA, that at 3 h obtained similar values to control, but at 12 h underwent again an augmentation. In the case of the *Hsf1*, it was down-regulated immediately after the injury in the HI group, while there was no difference in the rest of the time points between HI and control. Vascular endothelial growth factor was down-regulated just after the injury, then up-regulated at 3 h and down-regulated again at 12 h. No difference was observed between animals that underwent hypoxia-ischemia and those that did not receive any treatment and control group for the expression of *Hif1α.*

5.3.2. IMPLICATED ELEMENTS

We aimed to check the expression of different genes of some elements implicated in the brain injury, such as, synaptophysin (*Syp*), spinophilin or protein phosphatase 1 regulatory subunit 9B (*Ppp1r9b/Neurabin II*), chondroitin sulfate proteoglycan 4 (*Cspg4/NG2*), glial fibrilary acidic protein (*Gfap*) and vimentin (*Vim*).

5.3.2.1. SYNAPTOPHYSIN

Synapthophysin was down-regulated at 0 h in the HI (fold-change -0.73; $P<0.05$), RVT (fold-change -0.68; $P<0.05$) and DHA (fold-change -1.05; $P<0.05$) groups, but not in the RVT+DHA group (Figure 43A).

On the contrary, at 3 h there was not any significant difference among the experimental groups (Figure 43B).

However, 12 h after the hypoxic-ischemic injury, the expression of synapthophysin was down-regulated again in the HI group (fold-change -0.52; $P<0.05$) comparing with the control group. DHA and RVT+DHA underwent an increase in the expression comparing with HI group, showing similar values to control group (Figure 43C).

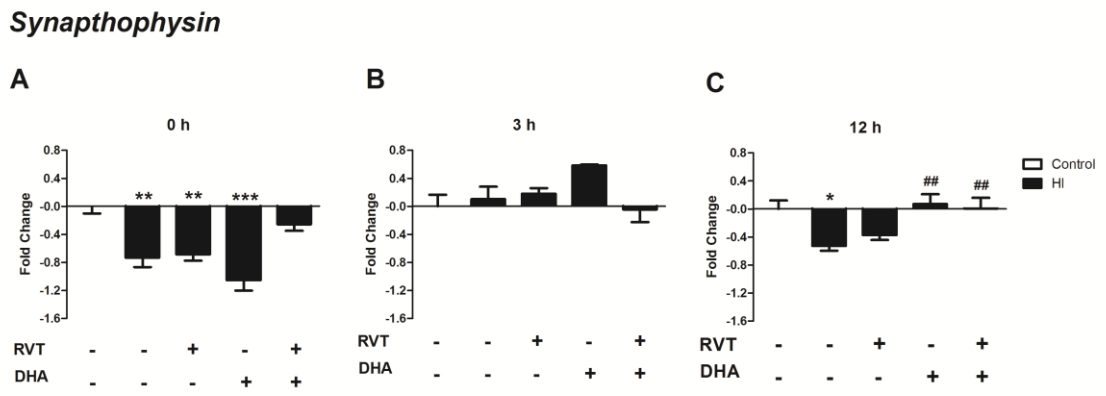


Figure 43: RT-PCR analysis of synapthophysin gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*P<0.05, **P<0.0005 or ***P<0.0001). The hash symbols denote the significance levels when compared to the HI group ($^{\#\#}P$<0.0005).

5.3.2.2. SPINOPHILIN

At 0 h, animals from HI group demonstrated a significant reduction of spinophilin mRNA quantity (fold-change -1.06; P<0.05), while animals pretreated with antioxidants did not (Figure 44A).

At 3 h, all the animals that underwent an hypoxic-ischemic injury showed an augmentation of the quantity, but only in the case of DHA groups this augmentation was statistically significant (fold-change 0.95; P<0.05) (Figure 44B).

On the contrary, at 12 h the expression of spinophilin was unaffected in any experimental group (Figure 44C).

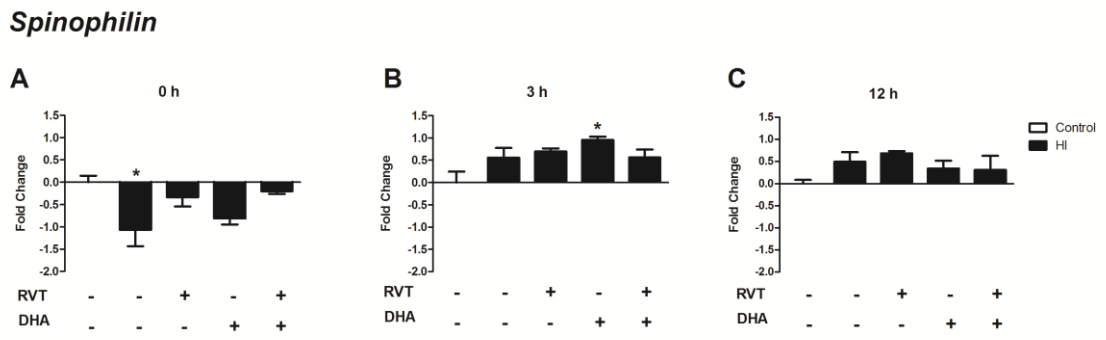


Figure 44: RT-PCR analysis of spinophilin gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (**P*<0.05). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05).

5.3.2.3. CHONDROITIN SULFATE PROTEOGLYCAN 4 (*Cspg4*)

No significant difference among groups in any time points was found (Figure 45).

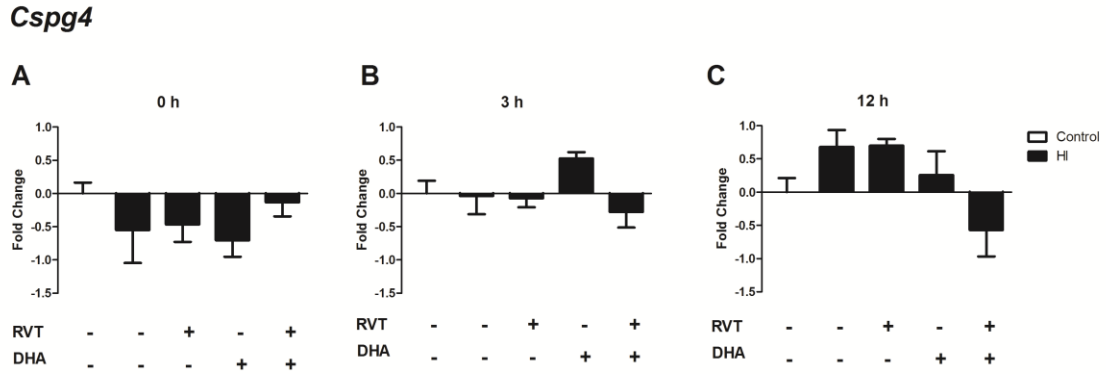


Figure 45: RT-PCR analysis of chondroitin sulfate proteoglycan gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD.

5.3.2.4. GLIAL FIBRILARY ACID PROTEIN (*Gfap*)

Following hypoxic-ischemic injury, the ipislareral side of the brain of the animals from the HI group underwent a significant reduction in the expression of the GFAP mRNA (fold-change -0.52; $P<0.05$) in comparison to the control (Figure 46A).

3 h after the injury, the quantity of *Gfap* mRNA was increased in the DHA group (fold-change 1.75; $P<0.05$) comparing with both control and HI groups (Figure 46B).

At 12 h, the expression of *Gfap* was up-regulated in RVT (fold-change 2.04; $P<0.05$) and DHA groups (fold-change 1.14; $P<0.05$) in comparison to control and HI groups, but no change was found between control and HI groups (Figure 46C).

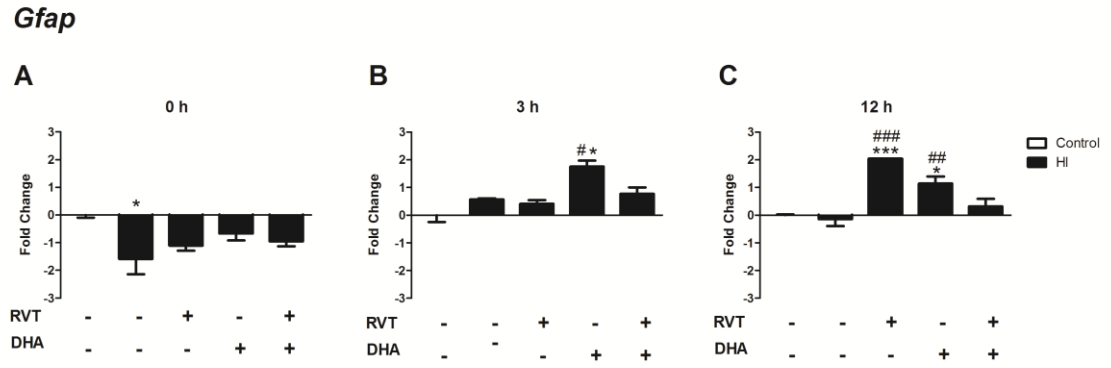


Figure 46: RT-PCR analysis of glial fibrilary acid protein gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*$P<0.05$ or ***$P<0.0001$). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P<0.05$, $^{\#\#}P<0.0005$ or $^{\#\#\#}P<0.0001$).

5.3.2.5. VIMENTIN

Immediately after the hypoxia-ischemia, only in the RVT+DHA group was a significant increase of the expression of this gene (fold-change 0.55; $P<0.05$) (Figure 47A).

At 3 h, the expression of vimentin was no altered among the groups (Figure 47B).

On the contrary, 12 h after the injury, there was a significant augmentation of the quantity of mRNA in HI (fold-change 1.29; $P<0.0005$), RVT (fold-change 2.08; $P<0.0001$) and DHA groups (fold-change 1.45; $P<0.0005$) in comparison to the control (Figure 47C).

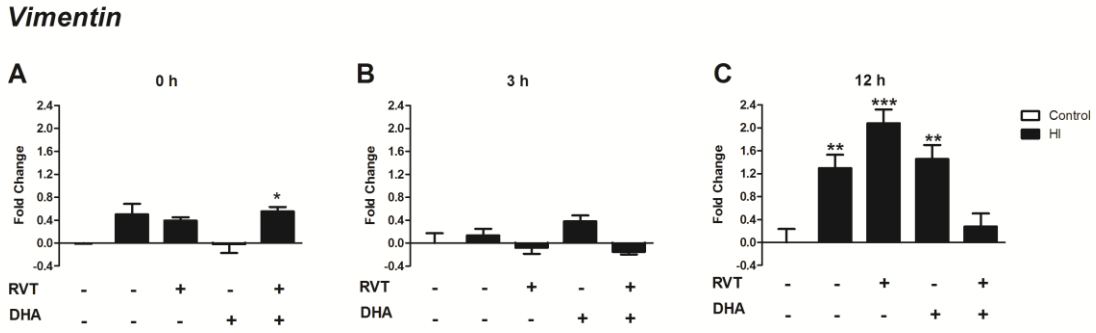


Figure 47: RT-PCR analysis of vimentin gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (**P*<0.05, ***P*<0.0005 or ****P*<0.0001).

In the case of the genes related with synapsis, immediately after hypoxic-ischemic brain injury there was a diminishment in the expression of both synaptophisin and spinophilin mRNA in the HI group, respect to the control. The immediate reduction of expression of spinophilin disappeared over the time, but not for synaptophisin, it was down-regulated again 12 h in the HI group. In the case of the animals pretreated with

antioxidants, they suffered a significant reduction just after injury in the expression of synaptophysin, but this down-regulation disappeared over time.

There was no meaningful changed in the expression of Cspg4 among groups in any time points. On the contrary, GFAP expression was diminished immediately after the injury in the HI group, and up-regulated in the DHA group at 3 h and 12 h. The expression of vimentin was up-regulated at 12 h in animals that underwent hypoxic-ischemic brain injury and in the animals that received resveratrol and DHA, but not in the ones that received the combination of both antioxidants.

5.3.3. OXIDATIVE STRESS

We wanted to evaluate the oxidative stress after the hypoxic-ischemic brain injury, so we took into account the expression of these genes: ciclooxigenase (*Cox2/ Ptgs2*), superoxide dismutase 2 (*Sod*) and nitric oxide synthase inducible (*Nos2*).

5.3.3.1. CICLOOXIGENASE 2 (*Cox2/ Ptgs2*)

At 0 h, the expression of *Cox2* was not altered in any of the experimental groups (Figure 48A).

However, 3 h after the hypoxic-ischemic event, the HI group underwent an important increase in the expression of this gene (fold-change 1.35; $P<0.05$) respect to the control. The group pretreated with DHA showed a reduction (fold-change 0.43; $P<0.05$) in comparison to HI group, demonstrating similar expression to control (Figure 48B).

On the contrary, at 12 h, the HI group suffered a diminishment in the quantity, but it was not significant. The treated groups demonstrated an augmentation in the expression of *Cox2* [fold-change: RVT (0.91), DHA (1.4), RVT+DHA (0.8); $P<0.0001$] comparing with HI group (Figure 48C).

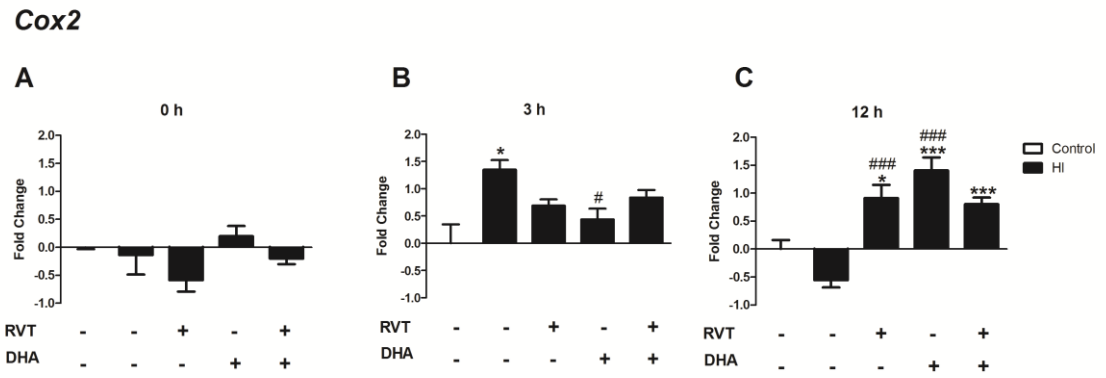


Figure 48: RT-PCR analysis of ciclooxigenase 2 gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (**P*<0.05 or ****P* <0.0001). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05 or $^{\#\#\#}P$<0.0001).

5.3.3.2. SUPEROXIDE DISMUTASE (*Sod*)

Following the induced brain injury, the *Sod* mRNA was down-regulated in all the animals that underwent hypoxia-ischemia, but only in HI (fold-change -0.35; *P*<0.0005), RVT (fold-change -0.36; *P*<0.0005) and DHA (fold-change -0.28; *P*<0.05) was significant (Figure 49A).

At 3 h, there was no any significant difference among the groups (Figure 49B).

In contrast, at 12 h, *Sod* was down-regulated in HI group, but not significantly. Meanwhile, it was up-regulated in the animals that received a single dose of any of the antioxidants [fold-change: RVT (0.16; *P*<0.0005); DHA (0.38; *P*<0.0001), RVT+DHA (0.26; *P*<0.0001)] in comparison to HI group (Figure 49C).

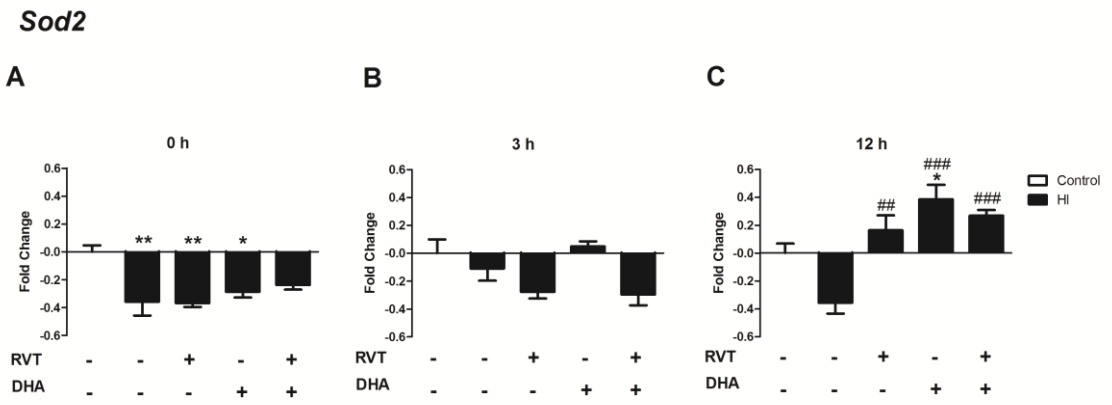


Figure 49: RT-PCR analysis of superoxide dismutase (SOD) gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*P < 0.05 or **P < 0.0005). The hash symbols denote the significance levels when compared to the HI group ($^{\#\#}P$<0.0005 or $^{\#\#\#}P$<0.0001).

5.3.3.3. NITRIC OXIDE SYNTHASE, INDUCIBLE (*Nos2*)

At 0 h, there was an up-regulation of *Nos2* in DHA group (fold-change 2.88; P<0.0001) comparing with control (Figure 50A).

At 3 h, the expression of *Nos2* was significantly increased in all the experimental groups underwent hypoxia-ischemia [fold-change: RVT (2.02; P<0.0001); DHA (2.43; P<0.0001), RVT+DHA (2.5; P<0.0001)], and especially in the animals that did not received any treatment (fold-change 2.98; P<0.0001) comparing with the control (Figure 50B).

At 12 h, the groups that received antioxidants suffered a significant augmentation [fold-change: RVT (2.89; P<0.0001); DHA (2.56; P<0.0005), RVT+DHA (2.5; P<0.0001)], comparing with both control and HI groups (Figure 50C).

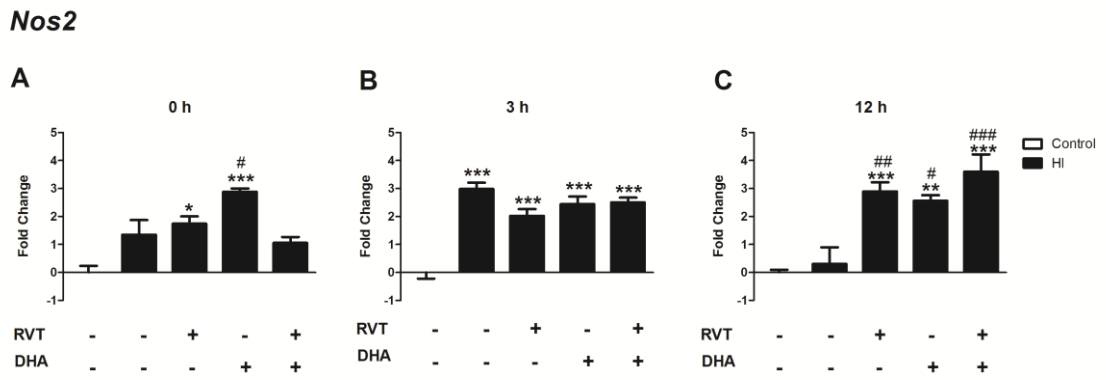


Figure 50: RT-PCR analysis of nitric oxide synthase inducible (NOS 2) gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*P < 0.05, **P < 0.0005 or ***P < 0.0001). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05, $^{\#\#}P$<0.0005 or $^{\#\#\#}P$<0.0001).

In regards to genes related with the oxidative stress caused by hypoxic-ischemic brain injury, our results show that there was an increase in the expression of *Cox2* and *Nos2* after 3 h and a decrease of *Sod* immediately after the insult in the HI group. On the contrary, animals pretreated with the antioxidants presented an up-regulation of these three mRNA: for *Cox2* and *Sod* 12 h after the event and for *Nos2* during the three time points.

5.3.4. INFLAMMATORY PATHWAYS

We wanted to evaluate if the inflammatory pathways were altered after the hypoxic-ischemic brain injury in the different experimental groups, so we quantified the expression of the interleukin-10, interleukin-6 and tumor necrosis factor-alpha.

5.3.4.1. INTERLEUKIN-10 (*Il10)*

At 0 h although there is an increase in the expression of *Il10* in the animals that underwent hypoxia-ischemia, this was not statistically significant (Figure 51A).

At 3 h, the *Il10* mRNA was up-regulated in HI (fold-change 2.98; *P*<0.0001) and RVT+DHA (fold-change 2.98; *P*<0.0001) groups (Figure 51B).

12 h after the insult, all the animals that underwent hypoxia-ischemia presented an increase in the expression of this gene [fold-change: HI (1.26; *P*<0.05); RVT (1.48; *P*<0.0005); DHA (1.21; *P*<0.05), RVT+DHA (1.27; *P*<0.05)] (Figure 51C).

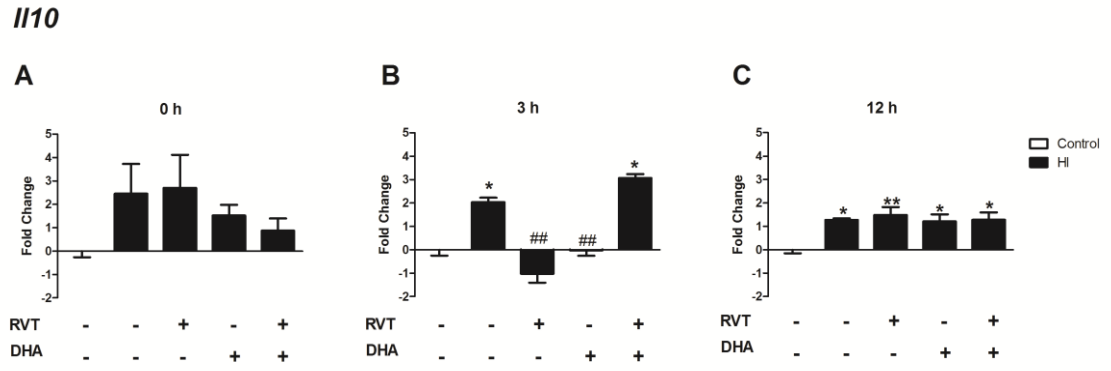


Figure 51: RT-PCR analysis of interleukin-10 gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (**P*<0.05 or ***P*<0.0005). The hash symbols denote the significance levels when compared to the HI group ($^{\#\#}P$<0.0005).

5.3.4.2. INTERLEUKIN-6 (*Il6*)

Immediately after the brain injury, the expression of interleukin-6 was up-regulated in HI (fold-change 3.57; *P*<0.0001), RVT (fold-change 2.38; *P*<0.05) and DHA (fold-change 2.95; *P*<0.0005) groups respect to the control (Figure 52A).

In the same way, at 3 h, this increase was maintained in HI (fold-change 2.01; $P<0.0001$), RVT (fold-change 2.49; $P<0.0001$) and RVT+DHA (fold-change 1.96; $P<0.0001$) groups (Figure 52B).

On the contrary, at 12 h there was no difference between HI and control groups, but the animals that received any of the treatment presented a higher expression [fold-change: RVT (4.26; $P<0.0001$); DHA (2.75; $P<0.0005$), RVT+DHA (2.85; $P<0.0005$)] (Figure 52C).

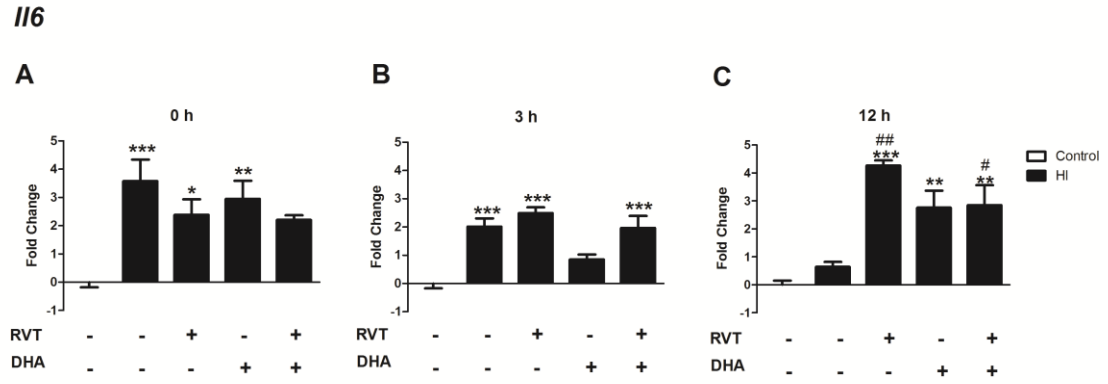


Figure 52: RT-PCR analysis of interleukin-6 gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*$P<0.05$, **$P<0.0005$ or ***$P<0.0001$). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P<0.05$ $^{\#\#}P<0.0005$).

5.3.5.3. TUMOR NECROSIS FACTOR ALPHA (*Tnfα*)

Immediately after the hypoxic-ischemic brain injury (0 h), there is a diminishment in the expression of *Tnfα* in all the animals underwent hypoxia-ischemia, especially in the HI group, but this reduction is was not statistically significant (Figure 53A).

3 h after the injury, *Tnfα* was up-regulated in the ipsilareral side of the brain of all the animals that underwent hypoxic-ischemic injury, comparing with the control group. The up-regulation was particularly evident in HI (fold-change 1.74; $P<0.0001$) and DHA groups (fold-change 2.28; $P<0.0001$) (Figure 53B).

At 12 h, there no was difference between the HI and control groups, but *Tnfα* was up-regulated in RVT (fold-change 1.83; $P<0.05$) and RVT+DHA groups (fold-change 1.9; $P<0.0005$) (Figure 53C).

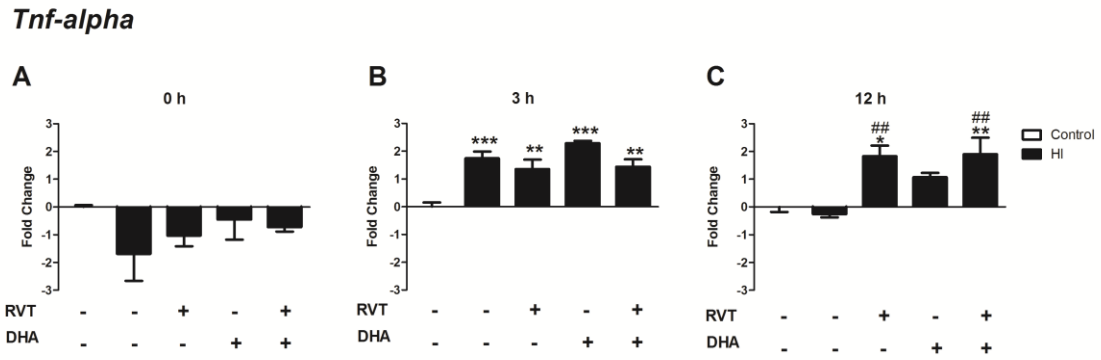


Figure 53: RT-PCR analysis of tumor necrosis factor alpha gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (**$P<0.0005$ or ***$P<0.0001$). The hash symbols denote the significance levels when compared to the HI group ($^{\#\#}P<0.0005$).

In regards to genes related with the inflammation, our results demonstrate that in the HI group there was a significant increase in the expression of *Il10* after 12 h, in the expression of *Il6* immediately after and 3 h later and in the expression of *Tnfα* 3 h after the injury comparing with the control group. Animals pretreated with antioxidants underwent an increase in the expression of mRNA *Il10* at 12 h, of *Il6* at any time point and of *Tnfα* at 3 h and 12 h.

5.3.5. DEATH PATHWAYS

To investigate if the death pathways in the ipsilateral brain of rats that underwent hypoxic-ischemic injury were altered, we quantified the expression of different genes related with cell death: BCL2-asociated X protein (*Bax)*, B-cell CLL/lymphoma 2 (*Bcl 2*) and caspase 3 (*Casp3*) by RT-qPCR.

5.3.5.1. B-CELL/ LYMPHOMA 2 (*Bcl-2*)

At 0 h, there was no difference among all the experimental groups (Figure 54A).

At 3 h, the animals pretreated with resveratrol demonstrated a significant decrease in the expression of *Bcl-2* (fold-change: -0.72, $P<0.05$) (Figure 54B).

In contrast, 12 h after the hypoxic-ischemic injury, in RVT group this gene was up-regulated (fold-change: 0.67, $P<0.05$) (Figure 54C).

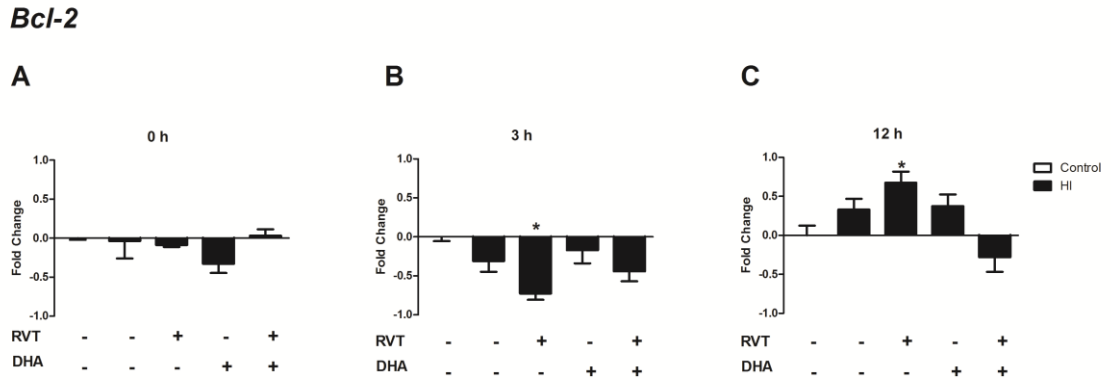


Figure 54: RT-PCR analysis of B-cell/ lymphoma 2 gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*$P<0.05$).

5.3.5.2. BCL-2-ASSOCIATED X PROTEIN (*Bax*)

At 0 h, the animals pretreated with RVT+DHA demonstrated a significant increase in the expression of *Bax* (fold-change: 0.34, $P<0.05$) comparing with control group (Figure 55A).

At 3 h, there was no difference among all the experimental groups (Figure 55B).

In contrast, at 12 h, the expression of *Bax* in RVT+DHA group was down-regulated in comparison to HI group, although there was any significant difference among the rest of the groups (Figure 55C).

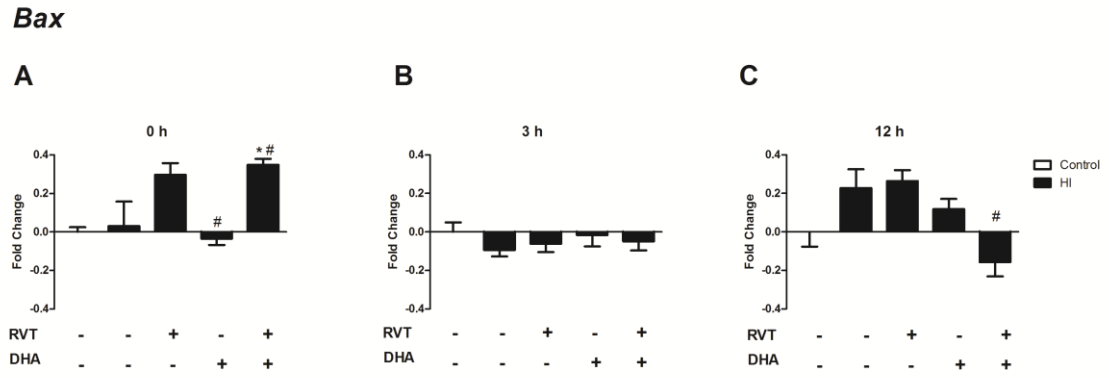


Figure 55: RT-PCR analysis of bcl-2-associated x protein gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*$P<0.05$). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P<0.05$).

5.3.5.3. CASPASE-3 (*Casp3*)

At 0 h, caspase-3 mRNA was up-regulated in RVT and RVT+DHA groups comparing with control; and down-regulated in DHA group comparing with HI group (Figure 56A).

At 3 h, there was no significant change in the expression (Figure 56B)

At 12 h, DHA and RVT+DHA group underwent a diminishment in the expression of caspase-3 comparing with HI group (Figure 56C).

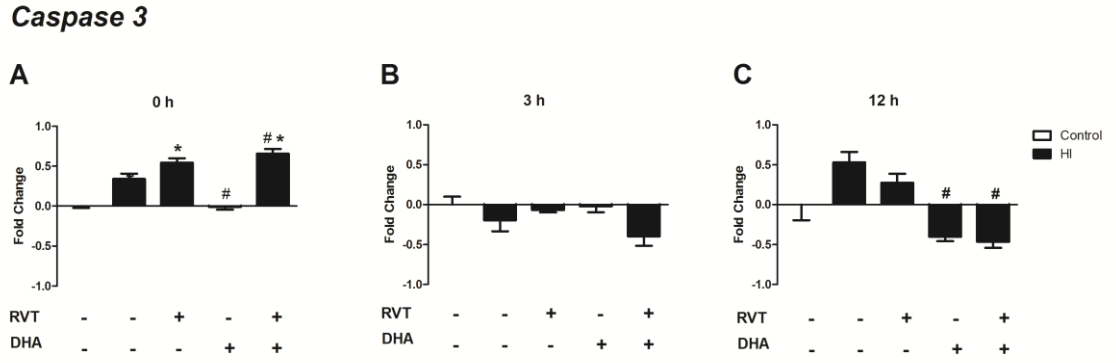


Figure 56: RT-PCR analysis of caspase-3 gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*P<0.05). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05).

5.3.5.4. CYTOCHROME C (*Cycs*)

Immediately after hypoxic-ischemic injury, there was no change in the expression of cytochrome c (Figure 57A).

At 3 h, RVT+DHA groups presented a significant reduction (fold-change: -0.27, P<0.05) comparing with control group (Figure 57B).

At 12 h, DHA and RVT+DHA groups, showed an increase in the expression comparing with HI group (Figure 57C).

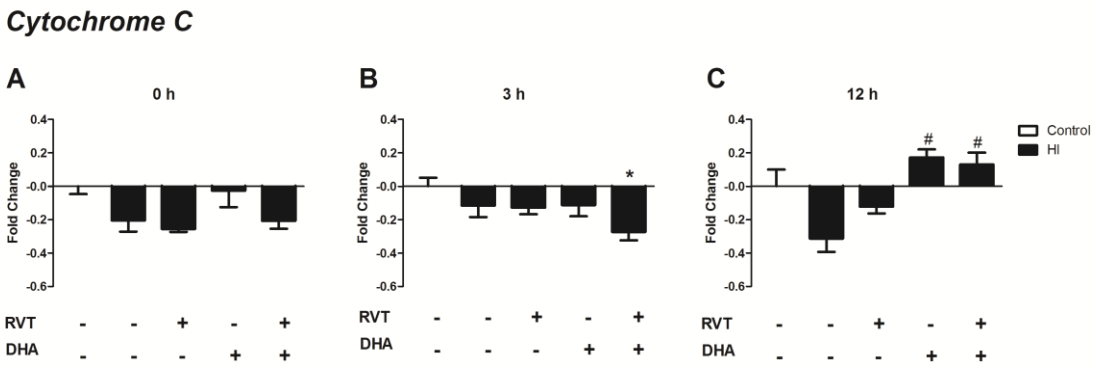


Figure 57: RT-PCR analysis of cytochrome C gene expression in the ipsilateral side of the brain of postnatal rats after hypoxic exposure at different time points after hypoxia-ischemia, (**A) 0 h, (B) 3 h and (C) 12 h**, in control, HI and animals pretreated with antioxidants (n=5, with triplicates). The graphical representation of the fold changes was quantified by normalization to the reference genes specific for each time point. Each bar represents the mean ± SD. Asterisks denote the significance levels when compared to the control group (*P<0.05). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05).

So in the general, there was no meaningful difference in any of the studied genes related to the cell death between HI and control animals in any time point. Animals pretreated with the combination of resveratrol and docosahexaenoic acid underwent an increase in the expression of *Bax* and caspase-3 immediately after the hypoxic-ischemic injury and a significant decrease at 12 h.

5.4. EVALUATION OF BEHAVIORAL IMPAIRMENT

On P90, we evaluated hypoxia-ischemia induced behavioral impairments by using the open field, hole-board, T-maze and novel object recognition tests in control, HI, RVT, DHA and RVT+DHA groups. We carried out these four behavioral tests in order to assess the effect of hypoxic-ischemic encephalopathy on the motor activity, the anxiety and neophobia, the long-lasting spatial and the exploratory behavior and the non-spatial working memory. Afterwards, we tested if administration of antioxidants could improve the behavioral impairments.

5.4.1. HYPOXIC-ISCHEMIC INJURY DID NOT ALTER MOTOR ACTIVITY

Animals that underwent hypoxic-ischemic injury did no present any significant difference when comparing with control group in the motor activity analyzed by the open field test in any of the evaluated parameters, such as the percentage time in the periphery (Figure 58A), percentage time in the center (Figure 58B), distance travelled in the periphery (Figure 58C), distance travelled in the center (Figure 58D), speed in the periphery (Figure 58E), speed in the center (Figure 58F), total speed (Figure 58G) and total distance travelled in the open field (Figure 58H). In the same way, no significant differences were found between control and HI animals pretreated with antioxidants. These results demonstrated that hypoxic-ischemic injury did not alter motor activity.

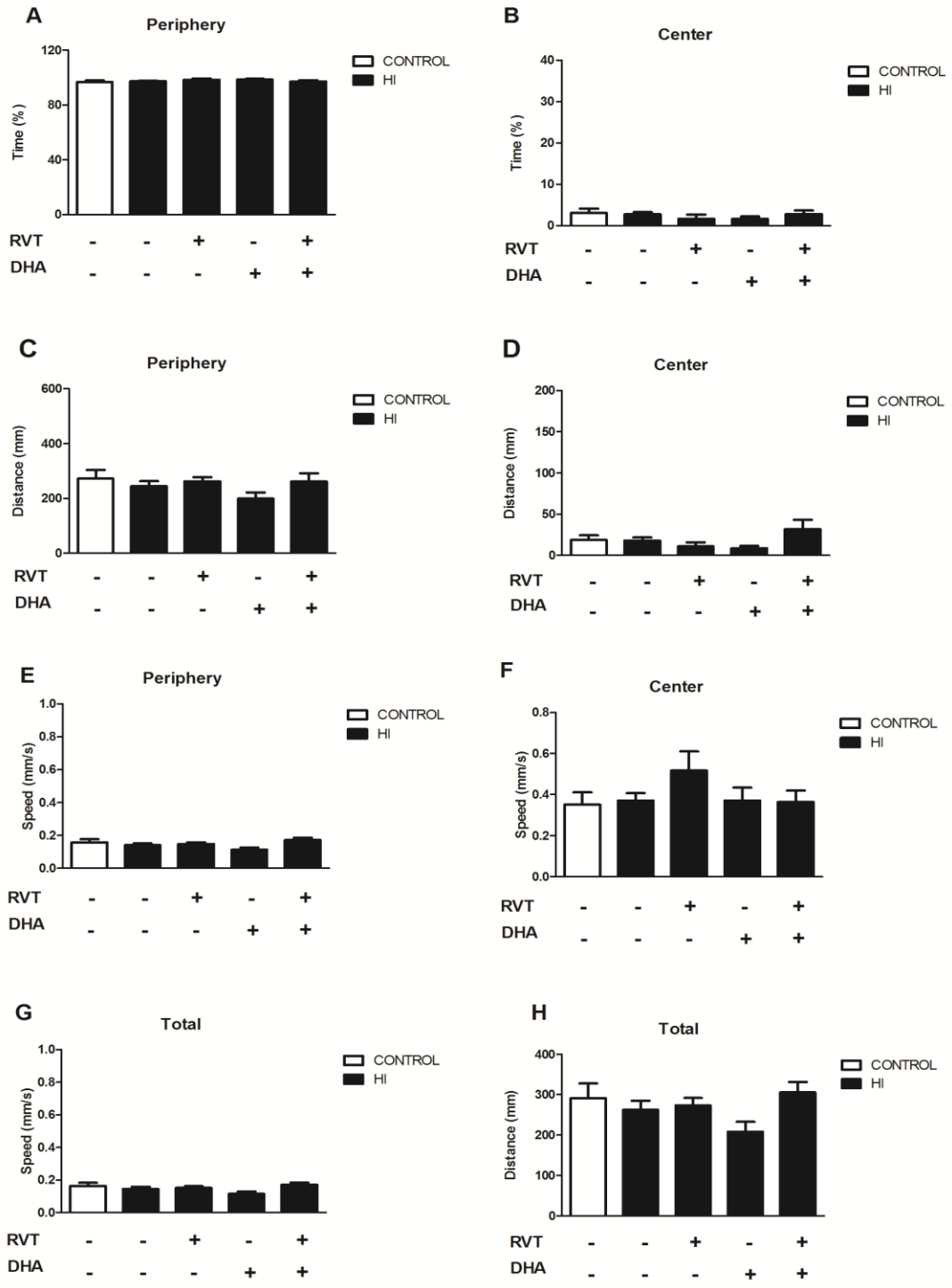


Figure 58: Spontaneous locomotor activity evaluation in the open field test performed at P90 in control (n=16), HI (n=14), RVT (20 mg/kg) (n=10), DHA (1 mg/kg) (n=14) and RVT+DHA treated animals (n=9). Evaluated parameters were (**A**) % time in the periphery, (**B**) % time in the center, (**C**) distance travelled in the periphery, (**D**) distance travelled in the center, (**E**) speed in the periphery, (**F**) speed in the center, (**G**) total speed and (**H**) total distance travelled in the open field.

5.4.2. ANTIOXIDANTS REDUCED ANXIETY AND NEOPHOBIA INDUCED BY HYPOXIA-ISCHEMIA

In the hole-board test (HBT), rats that underwent hypoxia-ischemia showed a statistically significant increase in the frequency of head-dipping behavior (P<0.005), when comparing to control animals (Figure 59A), suggesting an increase of the anxiety and neophobia. In contrast, pretreated animals presented similar values to non-ischemic, control rats, suggesting that antioxidants are able to reduced anxiety and neophobia related with hypoxic-ischemic injury, especially the group that received the combination of resveatrol and docosahexaenoic acid obtained better results.

No significant differences were found between groups in the hole-board test (HBT) for exploratory time (Figure 59B), although it can observe a tendency in the diminishment on the time spent in the exploration in the HI rats while pretreated animals showed similar values to control ones.

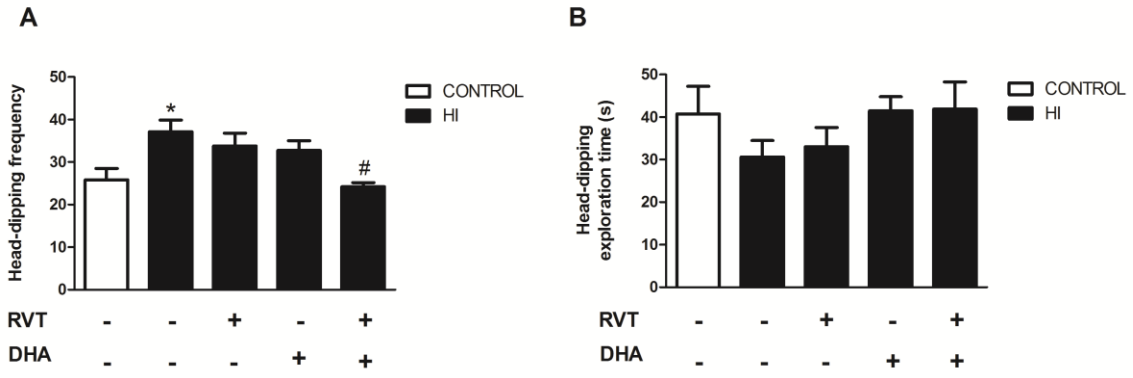


Figure 59: Effect of neonatal hypoxia-ischemia and antioxidant pretreatment on the anxiety and neophobia evaluated in the hole-board test performed at P90. (**A**) The frequency and (**B**) the time spent head-dipping into the holes were recorded in control (n=16), HI (n=14), RVT (n=10), DHA (n=14) and RVT+DHA treated animals (n=9). Asterisks denote the significance levels when compared to the control group (*P < 0.05). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05).

5.4.3. ANTIOXIDANT PRETREATMENT IMPROVED SPATIAL WORKING MEMORY IMPAIRMENTS

Spatial working memory was tested using a T-maze alternation task, where rats were tested for their performance in the maze recording the number of correct entries. At a 10 s delay interval (Figure 60A), no differences were found between groups.

In contrast, at a 40 s delay interval, HI animals made significantly (P<0.0001) fewer correct choices (Figure 60B) when compared to control animals. The administration of resveratrol and docosahexaenoic acid and particularly the combination of both reversed these changes and significantly increased the number of correct choices when compared to HI rats.

The results indicate a working memory dysfunction induced by hypoxia-ischemia, which is prevented by the acute administration of antioxidants before the ischemic event.

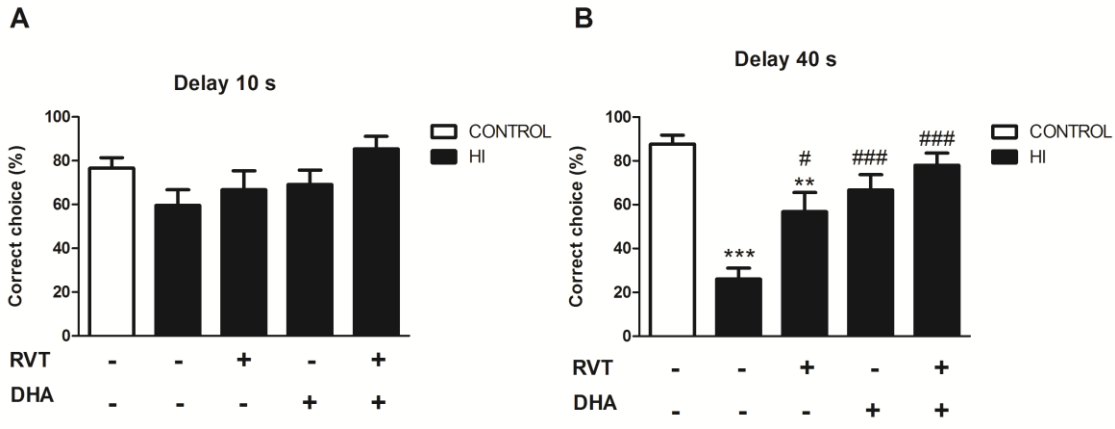


Figure 60: Effect of neonatal hypoxia-ischemia and antioxidant pretreatment on choice accuracy in the discrete-trial delayed spatial alternation task (T-maze) in adult animals on P90 in control (n=16), HI (n=14), RVT (n=10), DHA (n=14) and RVT+DHA treated animals (n=9). (**A**) Control and HI rats, as well as pretreated animals made a similar number of correct choices in the T-maze at 10 s delay. (**B**) In contrast, HI animals made significantly fewer correct choices after the 40 s delay. Antioxidants reverted the hypoxic-ischemic-impaired delayed alternation memory performance (percentage of correct trials). Asterisks denote the significance levels when compared to the control group (******P<0.005 or *******P<0.0001). The hash symbols denote the significance levels when compared to the HI group ($^{\#}P$<0.05 or $^{\#\#\#}P$<0.0001).

5.4.4. NON-SPATIAL WORKING MEMORY IMPAIRMENTS WERE PREVENTED BY ANTIOXIDANTS

Exploratory behavior and long-lasting non-spatial memory were evaluated by novel object recognition test and scored for investigation time of each object in the test session: in the familiar object (Figure 61A), in the new one (Figure 61B) and total time spent recognizing both objects (Figure 61C). No differences were found in the time spent in each object and neither in both object in any of the groups (Figure 61).

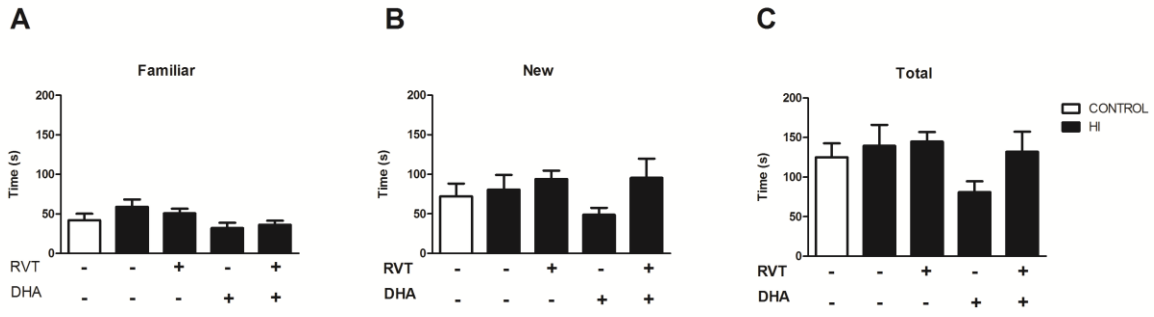


Figure 61: Time spent in the familiar object (A), in the new one (B) and total time spent recognizing both objects (C) on P90 in control (n=16), HI (n=14), RVT (n=10), DHA (n=14) and RVT+DHA treated animals (n=9). The histograms show the time spent (seconds), expressed as a mean ± SEM. No significant difference was found in the time spent in each object between any of the groups.

The discrimination index ([time in new object minus time in familiar object] / [time in new object plus time in familiar object]) was defined as the parameter for evaluation. Hypoxia-ischemia induced a significant (P<0.0001) profound decrease in the discrimination index when compared to control animals (Figure 62). In contrast, acute administration of the antioxidants fully reverted the effects of hypoxia-ischemia on novel object recognition, recovering discrimination index to control values, demonstrating that both antioxidants prevents non-spatial working memory deficits induced by hypoxia-ischemia, particularly the combination of both.

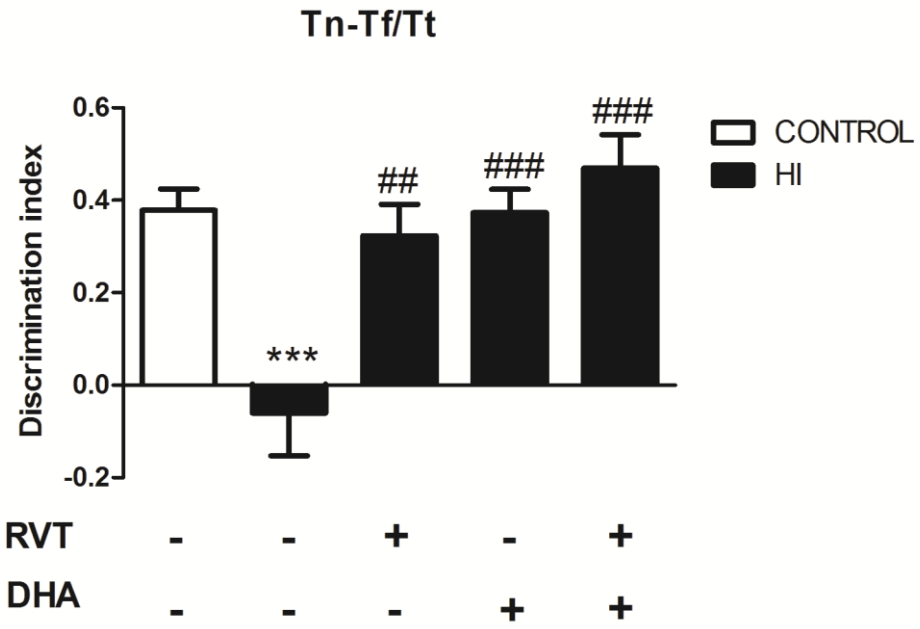


Figure 62: Effect of neonatal hypoxia-ischemia and antioxidant treatments in non-spatial working memory evaluated in a novel object recognition test on P90 in control (n=16), HI (n=14), RVT (n=10), DHA (n=14) and RVT+DHA treated animals (n=9)**.** The discrimination index ([time in new object minus time in familiar object] / [time in new object plus time in familiar object]) was defined as the parameter for evaluation, expressed as the mean ± SEM. HI animals displayed a decrease in discrimination index when compared to control animals that was fully reversed by acute administration of antioxidants, obtaining better results with the combination of RVT and DHA. Asterisk denotes the significance levels when compared to the control group (********P*<0.0001). The hash symbol denotes the significance levels when compared to the HI group ($^{\#\#}P$<0.005 or $^{\#\#\#}P$<0.0001).

5.5. EVALUATION OF NEURONAL CONNETION

5.5.1. ANTIOXIDANTS PREVENT CHANGES IN ANTEROGRADE AXONAL CONNECTIONS

For anterograde tracing experiments, biotinylated dextran amines (BDA) injections were made in left lateral of the cortex at the level of the somatosensory barrel cortex and auditory cortex on postnatal day 100 (Figure 63).

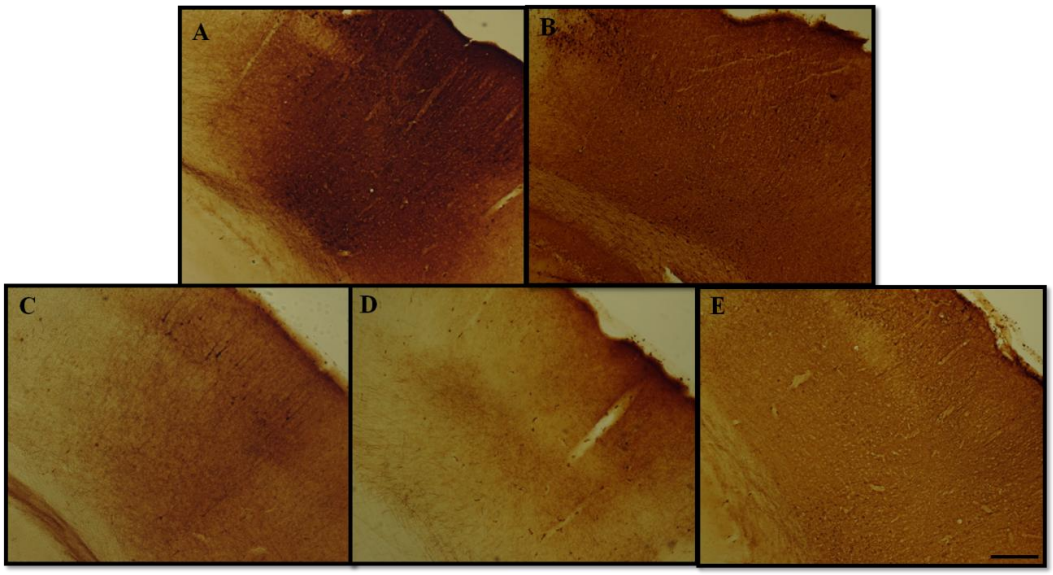


Figure 63: Representation in coronal slices of the extension of immunostained neurons of anterograde tracer injection in the cortex in adult animals on P90 in (A) control, (B) HI, (C) RVT, (D) DHA and (E) RVT+DHA treated animals (n=3). Scale bar: 40 µm.

Seven day later (P107) changes in the axonal connections of corticofugal neurons following neonatal hypoxic-ischemic injury were detected in the HI rats in the corpus callosum, there were fewer labeling fibers. In contrast, these changes were reverted with antioxidant pretreatment, demonstrating similar patterns to control rats (Figure 64).

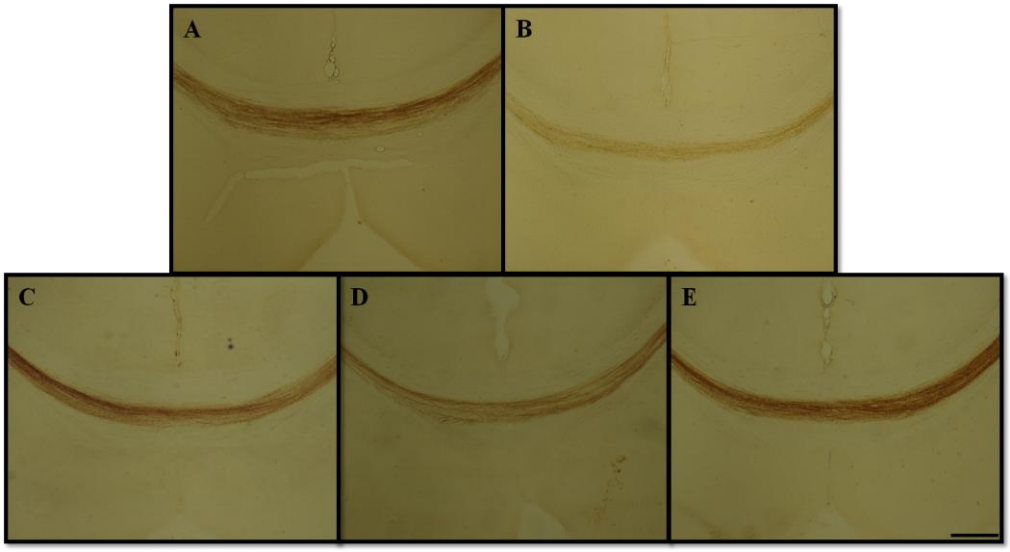


Figure 64: Representation in coronal slices of the extension of immunostained neurons in corpus callosum after anterograde tracer injection in cortex in adult animals on P100 (A) control, (B) HI, (C) RVT, (D) DHA and (E) RVT+DHA treated animals (n=3). Scale bar: 40 µm.

In the thalamus of the ipsilateral side of the brain, the animals that underwent the injury showed fewer immunostained neurons in comparison to the control group, while animals that received antioxidants, specially the group with the combination of both, presented almost the same pattern of staining of the control animals (Figure 65).

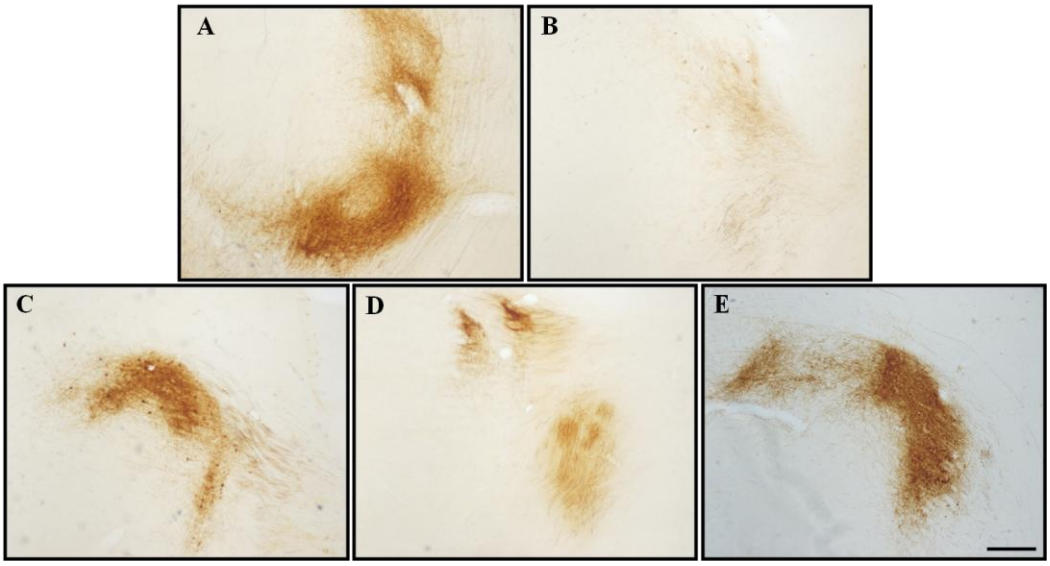


Figure 65: Representation of the extension of immunostained neurons in the thalamus of the ipsilateral side after anterograde tracer injection in cortex in adult animals on P100 (A) control, (B) HI, (C) RVT, (D) DHA and (E) RVT+DHA treated animals (n=3). Scale bar: 40 µm.

We found that the HI rats did not presented corticofugal neurons in the contralateral side of the cortex following neonatal hypoxic-ischemic injury, while pretreated animals showed similar patterns comparing with control ones (Figure 66).

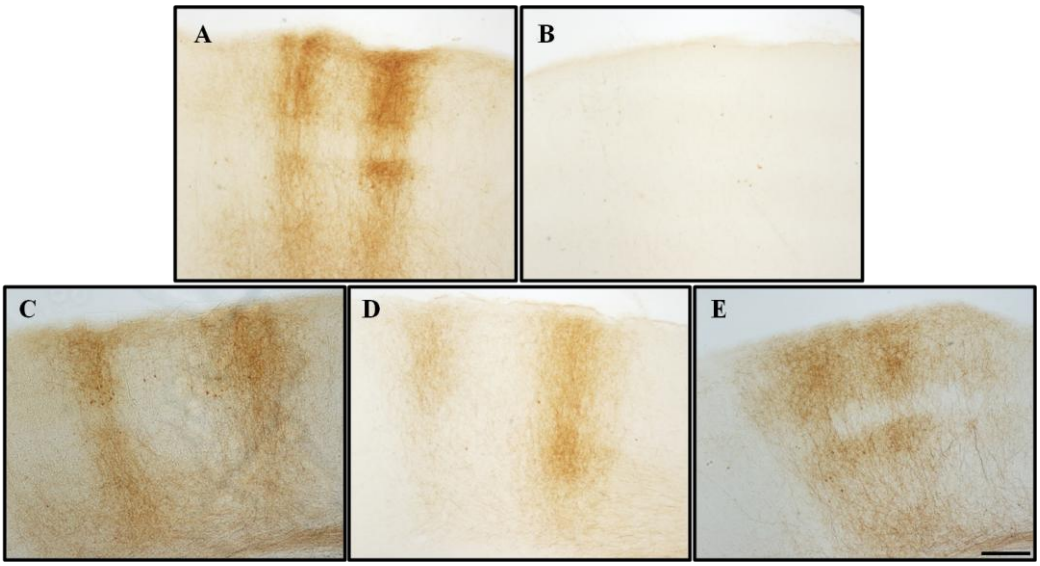


Figure 66: Representation of the extension of immunostained neurons in the cortico-cortical area of the contralateral side after anterograde tracer injection in adult animals on P100 (A) control, (B) HI, (C) RVT, (D) DHA and (E) RVT+DHA treated animals (n=3). Scale bar: 40 µm.

Taken together, our results indicate that the pretreatments with antioxidants were able to the changes in the axonal connections of corticofugal neurons in the corpus callosum, in the thalamus of the ipsilateral side and in the cortico-cortical area of the contralateral side induced by neonatal hypoxic-ischemic brain injury.

6. DISCUSSION

With an incidence of 2–6/1000 term births (De Haan et al., 2006), perinatal hypoxia-ischemia is a major cause of death and long-term disability worldwide, due to the frequent occurrence of irreversible brain damage and subsequent loss of neuronal function which lead to cognitive impairment and some motor dysfunction (Du Plessis and Volpe, 2002; Hamrick and Ferriero, 2003; Edward, 2010; Damoradan, 2014). Hypoxia-ischemia starts out a multi-faceted cascade of events that ultimately causes cell death and often damages the whole brain (Hilario et al., 2006). Everything starts with the reduction in oxygen and blood supply and finishes with the production of reactive oxygen species, which leads to oxidative stress, the increase in free cytosolic calcium concentrations and the fall in mitochondrial function triggering the activation of apoptotic pathways, DNA fragmentation and cell death (Alonso-Alconada et al., 2012).

The using of an appropriate animal model that simulates clinical neonatal hypoxic-ischemic encephalopathy is fundamental in order to clarify the pathogenesis of HIE and determine the effectiveness of therapeutic interventions (Yager and Ashwal, 2009; Park et al., 2015). This is why in this work, the Rice-Vannucci model of HIE in the 7-day-old Sprague-Dawley rat was used to induce cerebral hypoxia-ischemia, since it is a broadly used and standardized model for evaluating the therapeutic efficacy of neuroprotective strategies (Vannucci and Vannuci, 2005). In addition, the maturity of the rat CNS on P7 seems to be similar to that of human term babies (Hagberg et al., 1997; Volpe, 1998).

In the present doctoral thesis, we decided to evaluate the effectiveness of resveratrol and docosahexaenoic acid individually and in combination in an experimental model of neonatal hypoxic-ischemic brain injury at different time points. Firstly, a morphological study was done when rats were P14 (medium term) to evaluate the brain damage, focusing on the different cell populations of the CNS (neurons, astrocytes, oligodendrocytes and microglia). As the effectiveness of the treatments was proved, we analyzed mitochondrial state and ROS production by flow cytometry, as they are involved in the cascade triggered after hypoxia-ischemia, and the expression of different genes implicated in the injury immediately after, 3 h and 12 h after (short term). Finally, we carried out behavioral studies and neuronal tracing experiments in adulthood to evaluate the long-term effects of antioxidants on cognitive function (long term).

6.1. ANTIOXIDANT PRETREATMENT PROTECTED AGAINST BRAIN INJURY, CELL DAMAGE, ASTROGLIOSIS, MYELIN LOSS AND MICROGLIAL ACTIVATION

Neonatal hypoxia-ischemia not only causes brain damage and neurological deficits, but also decreases somatic growth. Accordingly, we found somatic growth retardation in HI rats at P14 compared to the control group, as well as Lara-Celador et al. (2012) and Alonso-Alconada et al. (2012). In contrast, antioxidant treatments significantly improved body weight, in agreement with the work of Revuelta et al. (2015).

In the neonatal hypoxic-ischemic rats used in the present study, volume reduction in the ipsilateral brain hemisphere was similar to that reported in previous studies (Wagner et al., 2002; Lubics et al., 2005; Lee et al., 2010). The tissue surrounding the cortical infarcted area appeared to be damaged, which is usual in this experimental model (Bona et al., 1998; Wagner et al., 2002; Spandou et al., 2005; Hobbs et al., 2008). Antioxidants were found to be potent protective agents that diminished tissue loss and consequently the infarct area, results in accordance with Zhang et al. (2015) and Mucci et al. (2015) for docosahexaenoic acid and West et al. (2007) and Karalis et al. (2011) for resveratrol.

Although theoretically all cell types are affected by hypoxia-ischemia, they do not respond in the same way. Depending on its severity and duration, brain injury may cause either infarction or selective neuronal death (Ferriero, 2004). The cells which are most sensitive to O_2 deprivation are neurons, which exhibit selective vulnerability (Northington et al., 2001; Hilario et al., 2005), whereas astrocytes are more resistant, so those that survived adjacent to the injured tissue are able to reestablish neuronal integrity (Barreto et al., 2011). Since neurons cannot survive in the brain without close interaction with astrocytes, in any region where astrocytes are injured neuronal survival will be affected and there will be no possibility for synacptic remodeling (Anderson et al., 2003). Therefore astrocytic functions can critically influence neuronal survival during ischemia (Raghubir, 2008). We found that pyramidal viable neurons of the hippocampus and parietal cortex underwent extensive degeneration following the injury, in agreement with different studies (Nakatomi et al., 2002, Lara-Celador et al., 2012).

On the contrary, results from both microscopic evaluation and histopathological scoring support the fact that pretreatment with antioxidants protects cells in these areas from injury, such as cell loss and swollen and damaged neurons.

Concerning astrocytes, these cells are known to respond to ischemic brain injury by increasing their expression of intermediate filaments, hypertrophying and proliferating, and often leading to glial scar formation (Takano et al., 2009; Honsa et al., 2014). Astrocytes can generate new cytoplasmic processes, an event associated with the accumulation of intermediate glial filaments such as GFAP (Panickar and Norenberg, 2005; Morken et al., 2014), which is rarely expressed by other cell types and it is used as a marker of reactive astrocytes (Eng et al., 2000). GFAP expression is required for normal function of astrocyte including maintenance of white matter and blood-barrier integrity. Following ischemia/reperfusion injury astrocytes become reactive and the expression of GFAP is enhanced (Eng et al., 2000; Raghubir 2008). In our model, we show that after the hypoxia-ischemia event, there is a modest increase in GFAP immunoreactivity. However, when antioxidants are administrated before injury, this up-regulation does not occur. This absence of astrogliosis in the pre-treated groups suggests that the magnitude of the impact of the ischemic stress had been reduced. In this sense, although partial astrocyte reactivity can confer neuroprotection by scavenging ROS and assisting with reconstruction from brain injury (Sizonenko et al., 2008), excessive astrogliosis can result in neuronal signaling impairment and deficiencies in pre-oligodendrocyte myelination (Faulkner, 2004; Sofroniew and Vinters, 2010).

White matter damage is also an important deleterious effect known to occur after hypoxic-ischemic injury in preterm children (Sanches et al., 2013). A widespread and complex inflammatory response to ischemia occurs which finally contributes to white matter injury (Alonso-Alconada et al, 2012), principally via oligodendroglia jeopardizing, since these cells are particularly susceptible to glutamate-mediated injury. An episode of hypoxia-ischemia causes rapid and severe damage to these particularly vulnerable cells via multiple pathways, such as over-activation of glutamate and ATP receptors, oxidative stress, and disruption of mitochondrial function (Mifsud et al., 2014). As a consequence, demyelination occurs, which has devastating effects on axonal function, transport, structure, metabolism, and survival (Fern et al., 2014). In summary, oligodendrocytes are one of the primary targets of the toxic effects of ischemia and therapeutic strategies should include their protection.

Since oligodendrocytes are the cells which produce myelin, a reduced staining for myelin basic protein is considered a hallmark of diffuse white matter damage in fetal rodents (Wang et al., 2007) and preterm infants (Inder et al., 2003). The pattern of MBP immunostaining which we found in the external capsule and striatum was different between groups and quantification of labeling allowed us to visualize the extent of these differences. Thus, the ratio of MBP staining in the ipsilateral and contralateral hemispheres revealed that there was a significant loss of MBP immunostaining in subcortical white matter at the level of both anatomical regions after hypoxia-ischemia. This loss of MBP was absent with antioxidant administration. These findings corroborate those of Karalis et al. (2011) and Revuelta et al. (2015) who observed how administration of resveratrol and DHA, respectively, led to a significant reduction in white matter damage, indicated mainly by the preservation of myelination.

Microglia, the resident immune cells of the nervous system, survey the brain for damage and infection, phagocythosing dead cells and debris. Microglia have also been implicated in synaptic remodeling during the development of the nervous system, when they are proposed to remove in appropriate synaptic connections through the process of phagocytosis. Furthermore, they are activated in many neurodegenerative diseases, although it is not yet clear if microglia are helpful or harmful in these conditions (Allen and Barres, 2009). Iba-1 is a microglia/macrophage-specific calcium-binding protein, which has the actin-bundling activity and participates in membrane ruffling and phagocytosis in activated microglia (Ohsawa et al., 2004).

Animals underwent hypoxic-ischemic injury showed an increase expression of Iba-1 seven days after the injury in the CA 1 and dentate gyrus areas of the hippocampus comparing with control animals. Li eta al. (2011) has previously demonstrated increased microglia formation after hypoxia-ischemia in neonatal and juvenile mice, both in the hippocampus (Qiu et al, 2007) as well as in the cortex and striatum (Zhu et al, 2009). In contrast, the pretreatment with antioxidants reduced the expression of Iba-1, showing that attenuation of the inflammatory response and microglial activation conferred neuroprotection in neonatal hypoxic-ischemic brain injury (Doverhag et al, 2010).

6.2. RESVERATROL AND DOCOSAHEXAENOIC ACID PREVENT THE MITOCHONDRIAL DAMAGE AND THE PRODUCTION OF REACTIVE OXYGEN SPECIES

Mitochondria play a fundamental role in the cascade triggered after hypoxic-ischemic injury, as mitochondrial respiration is suppressed (Hagberg et al., 2014). In the developing brain, lack of oxygen results in a depletion of cellular energy reserves, which trigger several pathophysiological responses, but there is a common convergence at level of the mitochondria (Thornton and Hagberg, 2015). This organelle has especial relevance in neurons because they have a high energy demand ad correspondingly high metabolic activity and under pathophysiological conditions, mitochondria can determine cells survival by overproduction of ROS, abnormal calcium homeostasis and release of apoptotic proteins (Sims and Anderson, 2002; Hong et al., 2004). This is why we decided to study the mitochondrial state by different and complementary forms.

Mitochondrial inner membrane integrity has been analyzed using the NAO marker. This fluorochrome accumulates in the mitochondria and binds to cardiolipin (Petit et al., 1992; García-Fernandez et al., 2004; Lara-Celador et al., 2012), which is essential in the mitochondrial respiratory chain. As cardiolipin is easily oxidized by the ROS, it causes a rearrangement in the mitochondrial structure, with the consequent reduction of NAO-binding ability (Ferlini et al., 1999; Alonso-Alconada et al, 2012). Our results indicate that the antioxidants maintain constant the number of cells with intact mitochondrial inner membrane and that those cells presented high quantity of cardiolipin 12 h after the injury, especially in the animals pretreated with DHA.

On the other hand, the presence of the electrochemical gradient in the mitochondria was assessed with Rh 123, which is retained in mitochondria in an amount proportional to the mitochondrial membrane potential (Goñi-de-Cerio et al., 2012), indicating its functionality. The loss of mitochondrial integrity or the opening of the permeability transition pore channel results in leakage of the probe from mitochondria and the consequent fluorescence decrease (Ferlini et al., 1995). When antioxidants were administered as pretreatment the percentage of cells with intact transmembrane potential and the fluorescence intensity was similar to controls, while HI animals showed a decrease in the number of Rh 123 positive cells in all the time points. This indicates that

the cells that are alive maintain their transmembrane potential, in fact, rhodamine 123 accumulates specifically in the mitochondria of living cells (Darzynkiewicz et al., 1981).

As we have mentioned, oxidative stress is considered to be a major contributor to hypoxic-ischemic brain injury. Intracellular ROS were detected using fluorocrome 2′,7′-dichlorofluorescein diacetate (DCFH-DA). This probe is cell-permeable and is hydrolyzed intracellularly to the DCFH carboxylate anion that is retained in the cell. Two-electron oxidation of DCFH results in the formation of a fluorescent product, dichlorofluorescein (DCF), which can be monitored by flow cytometry (Kalyanaraman et al., 2012). Our data show that although there was no significant difference in the number of cells producing ROS between the HI and control groups, there was an increase in the fluorescence intensity in the animals that underwent hypoxia-ischemia. That means that the cells which were producing these species were doing so more vigorously, particularly at 12 h. This might be partially due to the fact that mitochondria are both a source and a target of ROS (Zhao eta al., 2009), after the initial mitochondrial, an increase in the quantity of ROS production was observed to occur overtime. In contrast, this did not happen in case of the antioxidants which ameliorate the production at the first time points (0 h and 3 h), demonstrating their antioxidant effect. However, 12 h after the injury, only pretreatment with RVT in combination with DHA reduced ROS quantity.

Therefore, in addition to reducing the quantity of ROS production, we speculate that one of the ways these antioxidants might protect against hypoxic-ischemic damage may be by protecting mitochondrial inner membrane integrity and maintaining the membrane potential. Previous findings demonstrated that resveratrol exerts one of its neuroprotective effects by modulating mitochondria (Yousuf et al., 2009), inducing mitochondrial biogenesis against brain ischemic stroke (Dong et al., 2007) and preserving brain mitochondria functions after hypoxia-reoxygenation (Morin et al., 2003). In the same way, it is proved that DHA prevents paraquat-induced ROS production in dopaminergic neurons via enhancement of glutathione homeostasis (Lee et al., 2015) and that trigger apoptosis of colon cancer cells through a mitochondrial pathway (Zhang et al., 2015). Furthermore, our group has recently showed how resveratrol and docosahexaenoic acid reduced the damage caused by hypoxia-ischemia in the mitochondria in the brainstem (Revuelta et al., 2015).

6.3. HYPOXIA-ISCHEMIA ALTERED THE EXPRESSION OF SEVERAL GENES AT EARLY TIME POINTS AFTER THE INJURY AND THE ROLE OF ANTIOXIDANTS ON THESE MODIFICATIONS

In the present study we examined, in a prospective form, factors that may be involved in the development of hypoxic-ischemic encephalopathy in the neonatal brain and we found that the expression of some genes was up-regulated or down-regulated in response to the insult.

Immediately after the injury, the immediate early genes *c-Fos* and *Erg1* were significantly up-regulated in the HI group but at 12 h no difference was observed between control and HI group. Animals pretreated with antioxidants presented an important increased in the expression of *c-Fos* and *Erg1* at 0 h and 3 h as well, except in the case of DHA, that at 3 h obtained similar values to control. The protein encoded by *Egr1* is a nuclear protein which belongs to the *Egr* family of Cys_2His_2-type zinc finger proteins, and functions as a transcriptional regulator. Several studies suggest it has a role in neuronal plasticity. The transcription factor *c-Fos* is important in synaptic function and neuronal activity (Tsai et al., 2011), and its expression is involved in cellular proliferation, differentiation, and survival under hypoxia situations (Tulchinsky, 2000; Souza et al., 2014). The augmentation of *c-Fos* and *Erg1* are in accordance with the work of Gubits et al. (1993), who observed the induction of these transcription factors in ipsilateral forebrain at 1 h, 2 h and 3 h post-hypoxia; and with the study of Amén et al. (1994), who showed that in the ipsilateral hemisphere the expression of *c-fos* was very marked at 2 h. In addition, it is confirmed that the *Egr1* transcription factor is rapidly and transiently up-regulated by hypoxia and that *Egr1* is necessary for the trans-activation of the *Hif1α* promoter (Sperandio et al., 2009).

Hypoxia-inducible factor 1α, a transcriptional factor playing a central role in the maintenance of oxygen homeostasis (Li et al., 2014), has been largely studied for its role in cell survival in hypoxic conditions. The regulation of *Hif1* is a complex process and involves a number of molecules and pathways. Among these mechanisms a direct regulatory role of reactive oxygen species on *Hif1α* subunit has received a great deal of attention and the existing body of literature includes many contradictory findings

(Movafagh et al., 2015). In fact, the role of *Hif1* and its target genes, whether beneficial or detrimental, depends on such factors as severity and type of insult and age of the animal (Sheldon et al., 2014). Genetic reduction of neuronal *Hif1* results in a worsening of injury after neonatal hypoxia-ischemia, with a region-specific role for *Hif1* in the setting of neonatal brain injury (Sheldon et al., 2009); however, we did not find any difference regarding the expression of *Hif1α* mRNA among all the experimental groups. Li et al. (2014) have demonstrated the over-expression of the protein after neonatal hypoxic-ischemic brain injury, so we think that maybe there could be any transcriptional or translational regulation of this gene.

During hypoxia, *Hif1*α quickly binds the transcription regulatory region of the *Vegf* gene and induces its transcription and translation. Freshly produced *Vegf* activates endothelial cells and as a consequence new blood vessels are formed, which ultimately supply hypoxic areas with more blood and oxygen (Zhu et al., 2014). Our data prove that the vascular endothelial growth factor was down-regulated just after the injury, then up-regulated at 3 h and down-regulated again at 12 h. *Vegf* is the most potent stimulator of endothelial cells proliferation and angiogenesis. The strong angiogenic ability and vascular permeating effects of *Vegf* allow it to exert dualistic roles in stroke pathogenesis (Jones et al., 2001). In contrast, heat shock factor 1, an essential transcription factor in the response to cellular stress, is known to be induced and/or activated in response to hypoxia and ischemia (Chi et al., 2004). However, the role of *Hsf1* in ischemia-induced angiogenesis remains unclear; in fact in our data it was down-regulated immediately after the injury in the HI group, while there were no differences in the rest of the studied time points between HI and control. Gilby et al. (1997) found that the expression of HSP70 immunoreactivity was first observed at 24 h post-hypoxia. Additionally, nuclear accumulation of HSF1 protein and induction of *Hsp70* mRNA occurred dramatically in P26 neurons, but minimally in P7 neurons and moderately in microglial cells after hypoxia-ischemia (Sun et al., 2015). Thus, it could be possible that our studied time points are too early in time to observe any difference in mRNA expression.

Instantly after hypoxic-ischemic brain damage changes in the synaptic functions occur in the hippocampal pyramidal cells of neonatal rats (Zhao et al., 2012), since the synaptic transmission is rapidly attenuated by hypoxia-ischemia and after reperfusion, it briefly returns only to decrease as the secondary phase of energy depletion occurs (Rau

et al., 2012). We evaluate the synapsis by analyzing the expression of mRNA of synaptophysin, a calcium-binding protein that lies on the membrane of presynaptic vesicles, and spinophilin, a protein phosphatase 1 binding protein localized to dendritic spines. Immediately after hypoxic-ischemic brain injury there was a diminishment in the expression of both synaptophysin and spinophilin mRNA in the HI group, respect to the control. The immediate reduction of expression of spinophilin disappeared over the time, but not for synaptophisin; it was down-regulated again at 12 h in the HI group. While synaptophisin takes part in vesicles delivery, the development of nerve synapses and is involved in adjusting the plasticity of nerve synapses (Tarsa and Goda, 2002; Zhao et al., 2012), spinophilin modulates both glutamatergic synaptic transmission and dendritic morphology (Feng et al., 2000; Muhammad et al., 2015). Therefore, we could suppose that the synaptic transmission is reduced just after the brain injury in the Rice-Vannucci model. On the contrary, animals pretreated with antioxidants suffered a significant reduction just after injury in the expression of synaptophysin, but this down-regulation disappeared over time, and they did not present a significant difference in the expression of spinophilin, suggesting a protection in the synapsic processes.

Concerning the expression of the different elements implicated in the hypoxic-ischemic brain injury, we did not observe significant changes in the studied time points. The central nervous system contains abundance of astroglial cells which induce formation of neuronal synapses and support neurons structurally and metabolically. Astrocytes become activated by many pathological conditions such as neurotrauma, stroke, perinatal asphyxia or neurodegenerative diseases. One of the hallmarks of astrocyte activation is the up-regulation of the intermediate filament system composed of GFAP, vimentin and synemin (Järlestedt et al., 2010). In our case there was no meaningful changed in the expression of *Cspg4* among groups in any time points and, on the contrary, *Gfap* expression was diminished immediately after the injury in the HI group and was increased after 12 h in RVT and DHA groups. The expression of *vimentin* was up-regulated at 12 h in HI, RVT and DHA groups, but not in animals that received the combination of the antioxidants. Gubits et al. (1993) demonstrated that *Gfap* mRNA induction occurred predominantly in ipsilateral forebrain samples at 18 h and 24 h post-hypoxia. Similarly, Pimentel et al. (2011) observed a selective and delayed degeneration of hippocampal pyramidal neurons, GFAP-positive and vimentin-positive 8 days after hypoxic-ischemic injury. This findings evidence the extension of

the effects after hypoxia-ischemia, since the morphologic brain injury evolves over a period of several days to weeks after the insult (Nakajima et al., 2000), so maybe in these cases as well our studied time points were too early to observe any change in the expression of these genes.

As we have previously mentioned, there is little doubt about the fact that oxidative stress is a major contributor to ischemic brain injury (Wagner et al., 2004), since oxygen radicals are overproduced and the defense mechanisms are altered. In the present work hypoxic-ischemic brain injury results in an increase in the expression of *Cox2* and *Nos2* after 3 h, facts in accordance with the works of Forster et al. (1999) and of Nogawa et al. (1997), who observed an increase in the activity of inducible nitric oxide synthase and cyclooxygenase-2 after ischemia, respectively. Similarly, neither resveratrol nor DHA were able to reduce the expression of *Cox2* and *Nos2*. On the contrary, we found a decrease of *Sod* immediately after the insult in the HI group in comparison with control animals. The cells´ most important defense mechanism against the excessive production of three radicals is the concerted action of three enzymes: superoxide dismutase, glutathione peroxidase and catalase. SOD catalyzes the dismutation of the superoxide radical to hydrogen peroxide, and in our work the mRNA expression of this enzyme was reduced just after the injury in the HI group, indicating that the antioxidant defense mechanism was altered. Although immediately after the injury also animals treated with resveratrol and docosahexaenoic acid presented a down-regulation of *Sod* mRNA expression, 12 h after the damage these animals suffered an up-regulation comparing with the HI group demonstrating the ability of both components to increase the expression of this antioxidants enzyme.

After ischemic insults, the inflammatory cells in the brain are activated and then generate ROS via several enzyme systems to induce the expression of pro-inflammatory mediators including cytokines and adhesion molecules. Superoxide is generated via cyclooxygenase (Liu and Mccullough, 2013). The up-regulated expression of a large number of inflammatory factors and the activation of proteases become the primary pathomechanisms of nerve cell damage and blood–brain barrier destruction following cerebral ischemia. *Cox2*, *Hif*-independent hypoxia-inducible gene, was also up-regulated at the mRNA level in response to hypoxia (Ndubuizu et al., 2009). The participation of this inducible enzyme of inflammatory mediators *Cox2* in inflammatory reactions post-brain ischemia destroys the blood–brain barrier and causes cerebral

edema as well as changes in nerve functions. The expression of *Cox2* closely correlates with the severity of neuronal damage. *Cox2* catalyzes arachidonic acid to produce prostaglandin E2 with the latter promoting the syntheses of *Il1β* and *Il6*. The synthesized *Il1β* and *Il6* then promote the synthesis of COX-2, thereby forming a positive feedback loop. In this loop, *Cox2* serves as a stimulating factor that positively regulates inflammatory cells and promotes these cells to release a large amount of inflammatory factors (Wang et al., 2014). The first pro-inflammatory phase, which may cause damage in the tissue, involves the recruitment and activation of diverse immune cells and their interactions with parenchymal cells, promoting tissue repair and recovery (Nathan, 2002).

In fact, inflammation is an important contributing factor to CNS injury in neonates (Dammann and Leviton, 1997; Volpe, 2008; Stridth et al., 2013). Hypoxic-ischemic encephalopathy is associated with numerous factors, among which inflammatory reaction is an important mechanism. After brain ischemia, an increase in the expression of adhesion molecules and the activation of glial cells trigger inflammatory cascade reactions (Wang et al., 2014). Our data indicate that on the one hand, hypoxic-ischemic brain injury lead to an up-regulation of the pro-inflammatory molecule *Il6* just after and 3 h after the injury, and of *Tnfα* 3 h after the injury; and on the other hand, to an up-regulation of the anti-inflammatory *Il10* cytokine 12 h after the damage. The inflammatory cascade is characterized by a rapid activation of resident microglial cells and infiltration of peripheral leukocytes into the injured parenchyma (McRae et al., 1995; Kaur et al., 2013). Microglial contribution to secondary energy failure is thought to occur via production of pro- and anti-inflammatory cytokines such as interleukin *Il1β, Il6* and *Tnfα* (Szaflarski et al., 1995; Hagberg et al., 1996; Rocha-Ferriera and Hristova, 2015). Astroglial cells play a significant and sustained role in inflammation following brain injury, contributing to neuronal damage through the release of cytotoxic mediators, including cytokines, chemokines, reactive oxygen species, adhesion molecules, and matrix metalloproteins (Stigger et al., 2013; Chen et al. 2015). All pretreated animals underwent an increase in the expression of *Il6* at any time point and of *Tnfα* at 3 h and 12 h. Differently, all the animals pretreated with antioxidants underwent an increase in the expression of mRNA anti-inflammatory gene *Il10* 12 h after the injury and the ones receiving RVT+DHA also 3 h after the injury.

Regarding genes related to cellular death, the results indicate that there was no meaningful difference between HI and control animals in any of the studied at any time point. We speculate that there is no evidence of apoptosis at these time points because it is too early for the apoptosis cascade to start. We think that as we induce a moderate-severe hypoxic-ischemic brain injury, during the first hours the cells die by necrosis. In fact, primary energy failure takes place immediately after the hypoxic event, very abruptly, and corresponds at the cellular level to necrotic cell death, which is irreversible, and on MRI is noted as cytotoxic edema (Descloux et al., 2015). It is not clear when exactly the second energy failure occurs; most of the authors believe that it happens 6–48 h after an episode of hypoxia-ischemia (Dixon et al., 2015) without specifying the exact time point. According to Descloux et al. (2015) the second energy failure (associated with reperfusion injury) starts after 24 h, with a peak around 48 h, and after that, the brain energy level returns to near-normal values. This reperfusion stage is associated with the production of toxic substances, such as free radicals, and the activation of multiple cascades and transcription factors, with subsequent programmed cell death (Vannucci and Hagberg, 2004; Yager and Ashwal, 2009). Our group has demonstrated that the apoptosis was significant increased 24 h and 72 hours after the hypoxic-ischemic brain injury (Lara-Celador et al., 2012) in this experimental model in rats. The fact that the genes related with apoptotic pathway were not altered after hypoxic-ischemic injury support the results obtained by flow cytometry, where there was not mitochondrial inner membrane alteration until 12 h after the hypoxic-ischemic event.

Regarding the role of the antioxidants in gene expression alteration after the hypoxia-ischemia, the most significant findings of these prospective study are that the antioxidants are able to avoid the down-regulation of the expression of synaptophysin and spinophilin, suggesting a protection in synapsic processes, to up-regulate *Sod2* mRNA expression 12 h after the injury and to increase the expression of anti-inflammatory gene *Il10*. However, a more profound understanding of the molecular mechanisms of action of both components in the hypoxic-ischemic model will require further investigation. It would be interesting to evaluate the expression of these genes at protein level at longer time points by immunohistochemical and western blot techniques.

6.4. ANTIOXIDANTS IMPROVE NEUROLOGICAL FUNCTIONS AND CONFER LONG-TERM PROTECTION AGAINST CEREBRAL HYPOXIA-ISCHEMIA

One of the most significant findings of this study is the evidence that antioxidants were able to improve the long-lasting cognitive deficits induced by hypoxia-ischemia, probably via the protection and preservation of neocortical and subcortical brain areas (sensorimotor cortex, hippocampus and striatum), and in particular, of neuronal networks responsible for learning and memory. When exploring the potential neuroprotective effects of any treatment, it is essential to confirm that the apparent benefits are reflected in a long-term neurological status (Pazos et al., 2012). Thus, we performed different behavioral tests to evaluate locomotor activity, anxiety, and spatial and working memory at adulthood.

Curiously, we found no significant differences in locomotor activity, measured in an automated open field apparatus. These results indicate that hypoxia-ischemia did not exert any obvious effects on basic motor abilities, corroborating the findings reported by Damoradan et al. (2014), that there were no significant differences in locomotor activity between adult rats that underwent permanent bilateral common carotid artery occlusion and control ones. The fact that spontaneous motor activity was normal in injured rats could partly be due to the high degree of cerebral plasticity of the rats, differently from human neonates who do not present such high-level plasticity. Compensatory reorganization of vital functions following injury is possible and the contralateral hemisphere can functionally take over certain tasks of the injured hemisphere (Balduini et al., 2000; Lubics et al., 2005).

Bona et al (1997) explained it as an effect of aberrant corticospinal projections from the contralateral hemisphere that do not cross the midline. These fibers are spared when an ipsilateral lesion occurs and mask the damage until the contralateral cortex is lesioned in the same way. This was demonstrated by Barth and Stanfield (1990) after cortical ablation in neonatal rats. Thus, the aberrant ipsilateral corticospinal projection may provide the anatomical substrate through which the cortex carries out this recovery (Barth and Stanfield, 1990). Therefore, in general animals that underwent hypoxia-ischemia do not present evident functional deficits in motor activity, as injured rats

moved like control ones and they did not present abnormalities in locomotion or posture. However, several studies that have evaluated the effect in this experimental model on motor activity in the open field have presented contradictory results, in that while some of them found hyperactivity, others reported a reduction in general activity (Antier et al., 1998; Balduini et al., 2000; Lubics et al., 2005; Rojas et al., 2013, Xie et al., 2014). These discrepancies can likely be attributed to differences in the methods used to induce ischemia, in the way of carrying out the behavioral tests, and to differences in the age of the subjects at the time of the stoke or assessment.

Anxiety-like behaviors were assessed using the hole-board test. Rats that underwent hypoxia-ischemia exhibited an increased frequency of head-dipping while treated animals did not differ from controls, suggesting that hypoxia-ischemia is associated with long-term anxiogenic effects. Head-dipping behavior in the hole board test is considered to be a good index for evaluating the anxiety of rodents (Lee et al., 2014). Sab et al. (2013) assessed anxiety behavior by the elevated plus maze test in adult rats that had been affected by hypoxia-ischemia during pregnancy. They found that the time spent in open arms was extremely reduced in the hypoxia-ischemia group, showing increased aversion to open spaces. Xie et al. (2014) also measured anxiety by the distance moved in the open field test and demonstrated that hypoxic-ischemic rats showed increased motor activity and anxiety-related activity.

Impairments in reference and working memory after neonatal hypoxia-ischemia have been previously reported (Ikeda et al., 2001; Arteni et al., 2003, 2010; Pereira et al., 2007; Huang et al., 2009; Balduini et al., 2000; Simola et al., 2008; Matchett et al., 2007; Kadam et al., 2009; Pazos et al., 2012; Carloni et al., 2012). The present data have shown that the T-maze performance of neonatal injured rats was significantly worse at increasing delays, while it was stable with antioxidants. Mucci et al. (2015) have recently demonstrated how flaxseed, rich in DHA's precursor α-linolenic acid, consumption during gestation and lactation was able to improve spatial memory. Thus, hypoxic-ischemic rats showed deficits in the spontaneous alternation task and this demonstrates that our model induces deficits in cognitive behaviors that are hippocampal dependent as well as working memory dependent. It has been reported that these deficits are associated with cell loss in the hippocampus and cortex, since learning and memory depend on the appropriate working of both areas (Zhong et al., 2003; Choi et al., 2009).

The novel object recognition test assesses deficits in non-spatial working memory, especially visual episodic memory, and is based on the innate preference of rats to examine novel objects rather than familiar ones (Pazos et al., 2012; Dere et al., 2007; Broadbent et al., 2010). It is particularly indicated to evaluate the effects of drugs on memory (Dere et al., 2007), not involving primary reinforcements or stressful cues, such as food deprivation and/or electric shocks (Ennaceur and Delacour, 1998; Morrow et al., 2002; Simola et al., 2008). The results revealed that the discrimination rate was significantly lower in hypoxic-ischemic animals when compare with control rats. Pretreated animals obtained similar values to control ones; they spent more time with the new object than with the familiar one, so the treatment with this antioxidant prevented the memory deficit caused by the hypoxic-ischemic insult.

In conclusion, antioxidant administration before hypoxic-ischemic injury to newborn rats led to long-lasting neuroprotection, with the overall effect of preventing rats from experiencing greater functional damage. This cognitive improvement with respect to non-pretreated animals correlated with a significant reduction in morphological brain damage in the cerebral cortex and hippocampus, brain areas known to be essential for the acquisition and retention of spatial memory tasks. In fact, the cognitive dysfunctions induced by hypoxia-ischemia may result from abnormal communication within prefrontal-hippocampal networks, because synchrony and directed interactions between the prefrontal cortex and hippocampus account for mnemonic and executive performance (Brockmann et al., 2013).

Couriosly, our work demonstrates that the combination of resveratrol and docosahexaenoic acid was the pretreatment that showed better results in improving neurological functions and conferring long-term protection against cerebral hypoxia-ischemia. To date, therapeutic interventions against cerebral ischemia that only target a single pathogenic component have been largely unsuccessful, this may be the reason why the combination of both antioxidants is more effective, because both antioxidants could target different injured components.

The beneficial effects of resveratrol in cognition impairments have been reported in several studies. Resveratrol is neuroprotective and improves cognition in epilepsy in rats (Meng et al., 2014). It prevents impaired cognition induced by chronic unpredictable mild stress in rats in a stress model (Liu et al., 2014) and in a prenatal

stress model (Sahu et al., 2013); it improves learning and memory in normally aged mice (Zhao et al., 2013) and it has also been reported to improve cognition and reduce oxidative stress in rats with vascular dementia (Ma et al., 2013). Besides, this polyphenol has been shown to increase memory performance in primates and to increase hippocampal functional connectivity in older adults (Witte et al, 2014). More importantly, it has been shown to exhibit an important role in enhancing cerebrovascular and cognitive functions in humans (Wong et al., 2013).

In the case of DHA, although it has not been so researched, it is known that omega-3 supplementation lowers inflammation and anxiety in medical students, in fact the reduction in anxiety symptoms associated with n-3 supplementation provides the first evidence that n-3 may have potential anxiolytic benefits for individuals without an anxietydisorder diagnosis (Kiecolt-Glaser et al., 2011). In the same way, docosahexaenoic acid deficiency during development is associated with impairment in learning and memory, suggesting an important role of DHA in neuronal development, there are evidence that DHA promotes neuronal differentiation in rat embryonic hippocampal primary cultures (Calderon and Kim, 2004). Berman et al. (2009) have demonstrated that DHA pretreatment improves functionality at P14 pups, measured by the vibrissae-stimulated forepaw placing test, a readily quantifiable functional measure of injury to the sensorimotor cortex or striatum. Finally, Mucci et al. (2015) showed that this omega-3 improves spatial memory and lowered depressive behavior in a rodent model of neonatal hypoxic-ischemic encephalopathy.

6.5. ANTIOXIDANTS PREVENT CHANGES IN ANTEROGRADE AXONAL CONNECTIONS

Ipsilateral anterograde corticospinal tract tracing with biotinylated dextran amine showed that hypoxia-ischemia reduced BDA labeling of the contralateral cortex and of the ipsilateral thalamus, results in accordance with van Velthoven et al. (2010). Antioxidant treatments restored both contralateral and ipsilateral BDA labeling in those regions, indicating enhanced axonal remodeling. Moreover, we show that neonatal hypoxia-ischemia significantly reduced BDA labeling of the corpus callosum after BDA injection into the ipsilateral motor cortex (interaural distance of 5,40 mm and bregma of -3,60 mm). Importantly, BDA labeling in the corpus callosum of all antioxidant-treated HI animals were significantly increased compared with HI animals.

It has been widely accepted that the young nervous system possesses a greater capacity to recover from an injury than the adult nervous system. This potential has been attributed to the greater plasticity that exists in the neocortex of young animals as opposed to adults (Krageloh-Mann, 2004; Yang et al., 2007), and it seems that antioxidants help increasing this plasticity. Recovery of function has been related to the survival of neurons that would otherwise degenerate or to the synaptic reorganization at local or regional levels. We propose that memory recovery after the treatment with antioxidants may well be mediated by stimulation of axonal growth and/or remodeling leading to restoration of cortex integrity. In fact, we could relate the results from the tracing experiments with the behavioral tests in this regard: hypoxia-ischemia affects the somatosensorial area of the cortex, but not the motor area, so this may be the reason why the motor activity is not affected by the HIE while memory it is.

7. CONCLUSIONS

The results obtained in the present doctoral thesis suggest the next conclusions:

1. Antioxidants pretreatment with resveratrol and docosahexaenoic acid protected against brain injury, cell damage, astrogliosis, myelin loss and microglial activation.

2. These effects could be related to the prevention of the mitochondrial damage and the production of radical oxygen species.

3. Hypoxic-ischemic is correlated with the alteration of the expression of several genes immediately after the injury, while animals that received antioxidant administration did not show the same modifications in all the cases.

4. Antioxidants improve neurological functions and confer long-term protection against cerebral hypoxia-ischemia.

5. The administration of both resveratrol and DHA individually and in combination maintains the changes of the anterograde neuronal connetion caused by hypoxic-ischemic encephalopathy.

Doktorego tesi honetan eskuratutako emaitzek honako ondorio hauek iradokitzen dute:

1. Antioxidatzaileen pretratatmenduak burmuineko kaltearen aurka egiten du, zelulen kaltea leunduz, astrogliosi erreaktiboa gutxituz, oligondendrozitoen bideragarritasuna mantenduz eta mikrogliaren aktibazioa urrituz.

2. Efektu neurobabesle hauek mitokondrioaren kaltearen eta oxigenoaren espezie erreaktiboen ekoizpenaren murrizpenarekin erlaziona ditzakegu.

3. Hipoxia-iskemia neonatalak hainbat geneen berehalako espresioaren aldatzen du, zenbait kasutan antioxidatzaileak jasotzen dituzten animaliek aldaketa berdinak jasotzen ez dituztelarik.

4. Antioxidatzaileek funtzio neurologikoak hobetzen dituzte eta epe-luzeko neurobabesa eskaintzen dute hipoxia-iskemiaren aurka.

5. Bai erresberatrola eta azido dokosahexaenoikoak, indibidualki zein bien arteko konbinazioak, hipoxia-iskemiak eragindako konexio neuronal aurrerakoietako asaldurak murrizten dituzte.

8. THESIS

After the results obtained in this work we could suggest that the administration of resveratrol and docosahexaenoic acid both individually and in combination has a neuroprotective effect in hypoxic-ischemic brain injury in rats.

Our results provide novel evidence for the protective ability of resveratrol and docosahexaenoic acid when administered individually and in combination in the hypoxic-ischemic neonatal brain by reducing infarct volume, ameliorating cell damage, preserving myelin production, minimizing the astroglial reactive response and microglial activation, preventing mitochondrial inner membrane integrity and maintaining transmembrane potential, reducing the quantity of oxygen reactive species and improving the functional outcome. These antioxidants administrated before hypoxic-ischemic injury to newborn rats led to long-lasting neuroprotection, with the overall effect of preventing rats from experiencing greater functional damage. This cognitive improvement with respect to non-pretreated animals correlated with a significant reduction in morphological brain damage in the cerebral cortex and hippocampus, brain areas known to be essential for the acquisition and retention of spatial memory tasks.

We speculate that one of the mechanisms of resveratrol to protect against hypoxic-ischemic damage could be by protecting mitochondrial inner membrane integrity and maintaining transmembrane potential, as well as reducing ROS production. Further investigation for a better understanding of the role of these antioxidants in the hypoxic-ischemic model and their mechanisms of action is required. In this sense, the present study validates the high interest which is being invested in these molecules as promising therapeutic candidates for reducing ischemia-induced neurodegeneration in the neonate.

Lan honetatik lortutako emaitzen arabera, erresberatrola eta azido dokosahexaenoikoaren administrazioak, bai indibidualki ematerakoan zein modu konbinatuan, arratoietan hipoxia-iskemia neonatalak eragindako burmuin-kaltean efektu neurobasbesleak dituela onar dezakegu.

Gure emaitzek lehendabizikoz azaltzen dute erresberatrola eta azido dokosahexaenoikoaren administrazioak, bai indibidualki zein modu konbinatuan, hipoxia-iskemia neonatalak arratoietan eragindako burmuin-kaltearen kontra efektu babeslea duela, infartuaren bolumena txikituz, zelulen kaltea leunduz, mielinaren ekoizpena mantenduz, astrogliosi errekatiboa eta mikrogliaren aktibazioa urrituz, mitokondriaren barne-mintzeko osotasuna eta trasmintzeko potentziala mantenduz, oxigenoaren espezie erreaktiboen ekoizpena gutxituz eta epe luzeko kognizio asaldurak ekidituz. Antioxidatzaile hauek hipoxia-iskemiaren aurretik arratoiei emateak epe luzeko neurobabesa eskaintzen dute, konexio neuronalen galera ere saihestuz. Traturiko animaliek aurkezten dituzten kognizioaaren hobekuntza hauek, burmuineko garunazal parietalaren eta hipokanpoaren kalte-murrizketarekin erlazionaturik daude, memoriarekin guztiz loturik daudenak.

Guk espekulatzen dugu antioxidatzaile hauen mekanismo neurobabesletako bat mitokondriaren barne-mintzeko osotasuna eta trasmintzeko potentziala mantentzea dela, oxigenoaren espezie erreaktiboen ekoizpena gutxitzeaz gain. Hala ere, antioxidatzaile hauen ekintza mekanismoak hipoxia-iskemia eredu honetan zehazki zein diren hobeto ezagutzeko ikerketa gehiago beharrezkoak dira etorkizunean. Zentzu honetan, lan honek antioxidatzaile hauek hipoxia-iskemiak eragindako neuroendekapena murrizteko terapia aproposak direla balioztatzen du.

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10. SCIENTIFIC MANUSCRIPTS

NEUROPROTECTIVE EFFECT OF ANTIOXIDANTS IN NEONATAL RAT BRAIN AFTER HYPOXIA-ISCHEMIA

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Perinatal hypoxic-ischemic (HI) encephalopathy is one of the main causes of mortality and long-term disabilities in newborns. The adverse effect of birth asphyxia (deprivation of oxygen and glucose) in the brain can be devastating, resulting in death or severe neurological consequences such as mental retardation, learning disabilities, epilepsy and cerebral palsy. In spite of the advances in neonatology, The World Health Organization estimates that 4 to 9 million neonates suffer from birth asphyxia each year in the world, thus, the identification of new treatments is urgently needed. Nowadays, there are experimental evidences demonstrating that some antioxidant compound are effective in different animal models of HI brain injury, being able to reduce infarct volume and neuronal loss. In this review we will focus on the neuroprotective effect of resveratrol (RVT) and docosahexaenoic acid (DHA) in experimental HI encephalopathy and also discuss the possible synergy between different antioxidant agents.

Keywords: Hypoxia-ischemia, antioxidants, neuroprotection, resveratrol, docosahexaenoic acid.

1. INTRODUCTION

Neonatal hypoxia-ischemia and subsequent brain damage remains still as an important socio-sanitary problem, in spite of the advances in obstetric and neonatal care over the last decades. Perinatal asphyxia occurs in 1-10 of every 1000 live term births [1] and is considered the single most important cause of acute mortality and chronic disability in newborns worldwide [2,3]. Perinatal asphyxia may occur both prenatal (during birth and delivery) or in the immediate postnatal period. Data from epidemiological and neuropathological studies have identified several maternal and fetal risk factors, including placental abruption, cord compression, transplacental anaesthetic or narcotic administration, intrauterine pneumonia, severe meconium aspiration, congenital cardiac or pulmonary anomalies, birth trauma, obstructed airway, maternal opiated and congenital sepsis [1].

The result of a deprivation of oxygen and glucose to the brain give rise to death or severe neurological consequences such as cerebral palsy, mental retardation,visual and hearing impairment, learning and behavioural disabilities, attention deficits and hyperactivity and epilepsy [4-6]. The severity of neonatal encephalopathy depends on the intensity, duration and location of the insult [7]. The 15-20% of affected newborns will die in the postnatal period and an additional 25% will develop severe and permanent neuropsychological sequelae [8] with only a small percentage of infants with severe injury will survive without any handicap [9].

The clinical diagnosis of neonatal hypoxia-ischemia is based on two different criteria, one of them consists of evidence of neurological and cardio-respiratory depression (obtaining less than 7 in the Apgar score at 5 minutes after birth) and the other one consists of acidemia (defined as an arterial blood pH of less than 7), as the term asphyxia is defined experimentally as impaired respiratory gas exchange accompanied by the development of metabolic acidosis [10].

2. HYPOXIC-ISCHEMIC INJURY

The brain is the most metabolically active organ in the body, about the 95% of the consumed oxygen is reduced to ATP and the remaining 5% is released as radical oxygen species (ROS). The brain has a relatively high content of membrane lipids susceptible to oxidation and low antioxidant defence. Therefore, brain tissue maintains a fragile redox homeostasis and neurons are especially vulnerable to free radical damage. Under physiological conditions the antioxidant enzyme system balances redox homeostasis avoiding cell damage, but under ischemic conditions this system fails to protect cells from injury [11].

The developing brain is more susceptible than the adult one to HI events [12,13], due to its high concentration of unsaturated fatty acids, high rate of oxygen consumption, low concentration of antioxidants (vitamin E and ascorbic acid), high water content, low myelinization, availability of redox-active iron, an imbalance of antioxidant enzymes (catalase CuZn-superoxide dismutase-1 (SOD-1), mitochondrial superoxide dismutase-2 (SOD-2) and glutathione peroxidase (GPx)), and oxygen-induced vasoconstriction [7,14-17].

Insult from hypoxia-ischemia causes immediate neuronal injury and exhaustion of cellular energy stores, as the main cause of HI brain injury is the deprivation of glucose and oxygen supply, which initiates a multi-faceted cascade of biochemical events, as showed in Figure 1. Schematically two metabolic phases are recognized in the neurological damage, the first one is the primary energy failure due to the HI event and the second phase is consequence of the reoxigenation taking place some hours later.

Primary energy failure induces a decrease in oxidative phosphorylation and results in a change to anaerobic metabolism in order to maintain basic functions. Anaerobic metabolism, which is energetically inefficient, leads to a rapid depletion of ATP, accumulation of lactic acid and failure of ion pumps, resulting in a massive entry of sodium, calcium, and water into the cells, The membrane depolarization results in an excessive release of excitatory neurotransmitters, mainly glutamate which activates N-methyl-D-aspartate (NMDA) receptors triggering excitatory cascade and promoting death [18]. The secondary phase of damage, which occurs from 6 to 48 hours after the primary event, is related to the reoxigenation, involving a mitochondrial dysfunction that extends and increases the reactions from primary phase [19]. The massive increase in free cytosolic calcium concentration induces the production of nitric oxide (NO) by activation of neuronal nitric oxide synthase (nNOS) and the generation of free radicals (as we will see below in section describing oxidative stress) This results in the degradation of cellular lipids (by activation of phospholipases), proteins (by activation of proteases), and DNA (by activation of nucleases) [20-26].

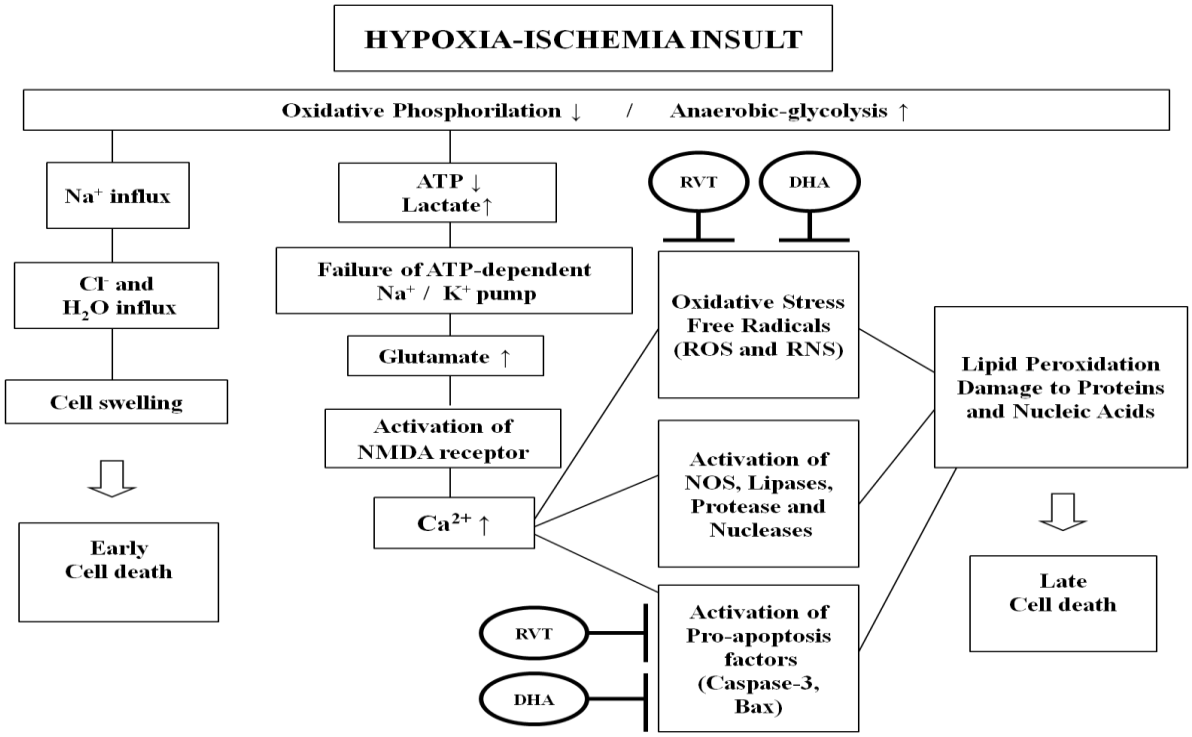

Fig 1 Biochemical mechanisms involved after neonatal hypoxia-ischemia and the main effects of the antioxidants Resveratrol (RVT) and Docosahexanoic acid (DHA).

The combined effects of cellular energy failure, acidosis, glutamate release, intracellular calcium accumulation, lipid peroxidation, and nitric oxide neurotoxicity provoke, in many cases, the death of the cells, either by necrosis or apoptosis [27,28], a divergence that will depend on the severity of the insult, the maturational state of the cell or the brain region affected, among others. Necrosis usually occurs in regions with more damage, while apoptosis appears in regions with moderate injury [32]. On the other hand, the first usually appears immediately after the insult, while the later can persist for at least 7 days after injury [29-31]. Mitochondria play a central role in the apoptotic process, as the permeabilization of its outer membrane can trigger the release of the apoptosis-inducing factor and the cytochrome C, which leads to the cleavage of caspases 9 and 3, two of the major effectors of delayed cell death [33].

3. OXIDATIVE STRESS

Oxidative stress (OS), which is defined as an imbalance between oxidant and antioxidant factors, contributes to neural cell damage following HI brain injury. There is little doubt about the fact that OS is a major contributor to ischemic brain injury [34], because it is an important consequence of the neurotransmitter-mediated toxicity following perinatal asphyxia. Oxidative stress occurs when the equilibrium between pro-oxidants and antioxidants is lost and there is a consequent excessive production of reactive oxygen species (ROS). ROS, defined as highly reactive due to the presence of unpaired electrons, play an important role in some physiological processes but are also implicated in a significant number of diseases, where they mediate cellular damage and compromise cell function [35, 36]. ROS regulate neuronal signalling in both the central and the peripheral nervous system, and mainly generated by microglia and astrocytes, modulating synaptic transmission and non-synaptic communication between neurons and glia. Superoxide anion, hydrogen peroxide and NO all participate in the communication between neurons and glia in the hippocampus [37].

ROS include radical species, such as superoxide and hydroxil radical and non-radical toxic species, such as singlet oxygen and hydrogen peroxide [38]. Free radicals (FRs) are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules. Once formed these highly reactive radicals can start a chain reaction: FRs react quickly with the nearest stable molecule to capture the electron they need to gain stability and the injured molecule loses its electron, becoming FR itself. Their main danger comes from the damage they can do when they react with important cellular components such as nucleic acids, cell membranes and mitochondria, resulting in subsequent cell death [16]. Apart from ROS, we can also find reactive nitrogen species (RNS). During hypoxia-ischemia, the excessive increase in free cytosolic calcium concentration activates nNOS which in turn catalyzes the synthesis of nitric oxide [39,40]. NO is constitutively produced by endothelial NOS (eNOS) in endothelial cells and by nNOS in astrocytes and neurons. An inducible isoform of NOS (iNOS) also produces NO in response to cellular stress, which initiates neuronal damage when converted to secondary RNS that facilitate nitration and nitrosylation reactions. In fact, NO itself is unreactive with most biological molecules and plays an important role in pulmonary, systemic, and cerebral

vasodilation, but becomes toxic when converted to secondary RNS [41]. NO can combine with superoxide radicals to produce peroxynitrite that spontaneously decomposes to form hydroxyl radicals, nitrogen dioxide and nitronium ion. In this way, NO can augment significantly the toxicity of superoxide radicals by converting them into highly potent radicals which in turn cause neuronal cell damage by reacting with and damaging most cellular targets including lipids, proteins and DNA [23,24].

ROS are implicated in brain injury after perinatal asphyxia. Plasma malondialdehyde (MDA), a stable product formed out of lipid peroxidation, and nitrate/nitrite levels were significantly higher in infants with hypoxic ischemic encephalopathy, indicating the possible role of free radical injury in brain damage [42]. During reoxygenation, the excessive increase in ROS production rapidly overwhelm antioxidant defences, causing further tissue damage: lipid peroxidation, protein denaturation, inactivation of enzymes, DNA damage, release of calcium from intracellular stores and damage to the cytoskeletal structure [43]. This cellular damage can lead to necrosis, apoptosis and autophagy. Furthermore, the rapid restoration of blood flow increases the level of tissue oxygenation and accounts for a second burst of ROS generation, which cause reperfusion injury [44]. The polyunsaturated fatty acids of the biological cell membrane are extremely vulnerable to free radical–induced peroxidation [42], this is why the cerebral vasculature is a major target of OS and it plays a critical role in the pathogenesis of ischemic brain injury following a cerebrovascular event.

4. ANTIOXIDANT THERAPIES

In order to prevent free radical damage, the body has developed an antioxidant system of defence, able to handle and scavenge FRs. This antioxidant system is composed, on the one hand, of antioxidant enzymes, such as SOD, GPx, catalase, and on the other hand, of antioxidant compounds (vitamins A, C, E, beta-carotene, lipoic acid, glutathione and ubiquinones). Thus, antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged, by removing free radical intermediates and by inhibiting the oxidation of other molecules. They do this by being oxidized themselves, so antioxidants are often reducing agents

such as thiols, ascorbic acid or polyphenols.

As we have seen the brain is highly at risk of free radical-mediated injury because neuronal membranes are rich in polyunsaturated fatty acids and because the human newborn has a relative deficiency in brain SOD and GPx. Since antioxidant defence system do not accelerate in maturation until late third trimester of pregnancy [45], exogenous antioxidant therapy would be helpful in order to prevent cellular damage if perinatal asphyxia occurs. In this review we will focus on the neuroprotective effect of resveratrol and docosahexaenoic acid in experimental HI encephalopathy and also discuss the possible synergy between different antioxidant agents.

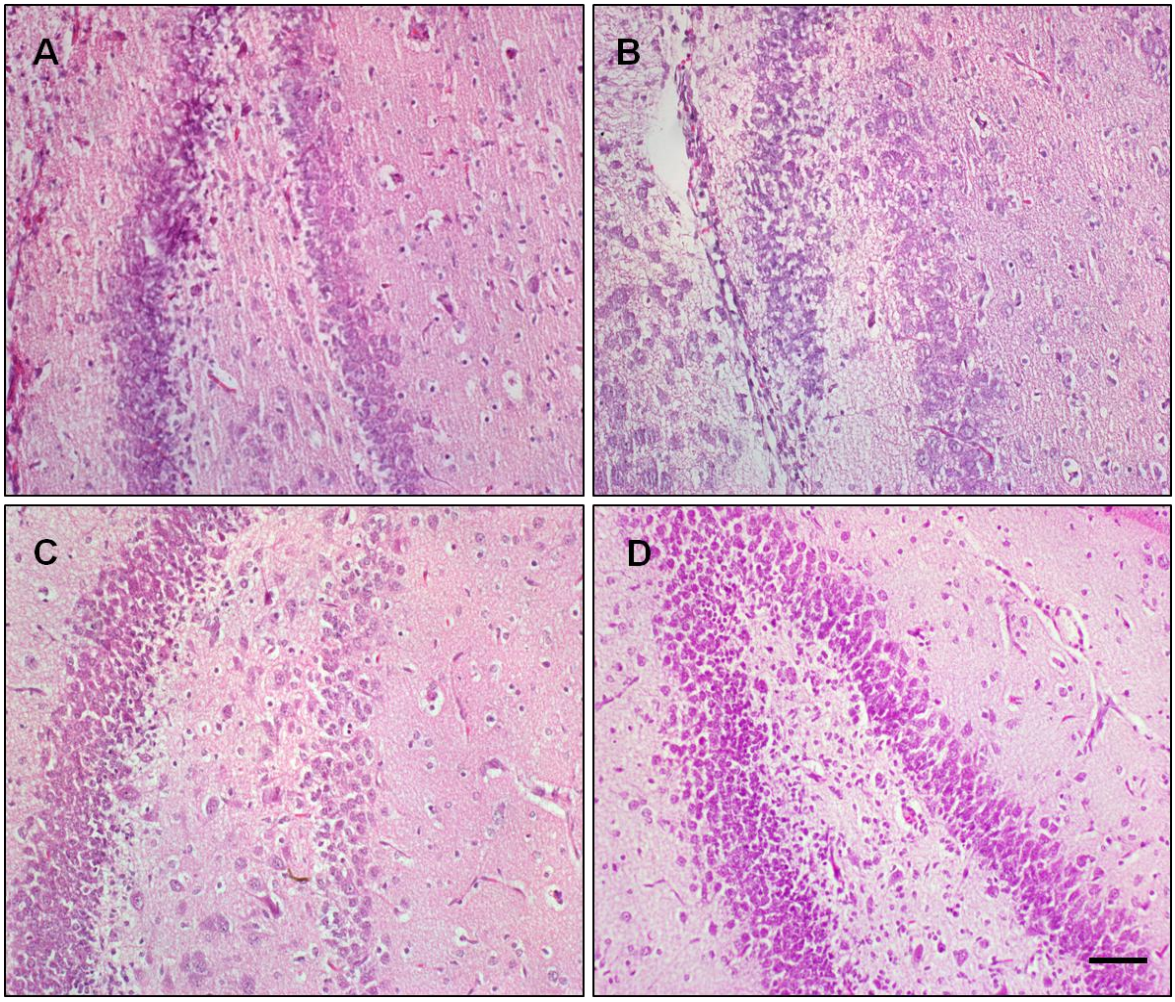

Fig 2 Representative light microphotographs of hematoxylin-eosin stained brain sections obtained from dentate gyrus in the hippocampus of neonatal rats. A) Control; B) HI rat; C) HI rat with RVT; and D) HI rat with DHA. Bar: 40 μm.

4.1 RESVERATROL

Resveratrol (3,5,4′-trihydroxystilbene) is a non-flavonoid polyphenolic compound consisting of two aromatic rings attached by a methylene bridge, which is produced by 72 different plant species, including grapevines, pines, legumes, peanuts, soybeans and pomegranates [47]. The most common dietary source of resveratrol is red wine, and it is thought to be an influential factor in the French Paradox, a term describing the observation that the French population has a very low incidence of cardiovascular disease, in spite of a diet high in saturated fats [48].

This natural polyphenol seems to play an important role in neuroprotection in models of neurodegeneration (Alzheimer's, Parkinson's or Huntington's disease), ischemia and brain and spinal cord injury [49,50]. The neuroprotective effects of RVT result from its antioxidant activity due to its stilbene structure with two phenol rings, that allows it to scavenge a variety of free radicals, including lipid peroxyl and carbon-centered radicals, reactive oxygen species, and because of its capacity to induce the expression of several antioxidant enzymes, such as SOD and GPx. Recent studies have revealed that resveratrol modulates the gene response related to redox pathways [51].

There are several evidences that demonstrate the efficacy of RVT in hypoxic-ischemic brain injury in the neonatal rat. Resveratrol significantly reduces brain injury, protecting against tissue loss measured at 7 days after the injury, by preserving neocortical and subcortical brain areas (sensorimotor cortex, hippocampus and striatum) and also neuronal networks responsible for learning and memory functions. In this way it ameliorates HI-induced short- and long-term behavioural deficits and significantly improves motor performanced, and what is more, animals treated with resveratrol presented more cells morphologically well preserved than in the HI group [52-54]. It also maintains myelination, revealed by myelin basic protein (MBP) immunolabelling and Luxol Fast Blue (LFB), which means a significant reduction in white matter damage [52]**.** Resveratrol plays a neuroprotective role in HI brain damage through its anti-apoptotic effects, by means of decreasing the expression of Bax, Caspase-3 and the ratio of Bax/Bcl-2 and also increasing Bcl-2 expression. It also reduces the calpain activation, suggesting that it works as a generally neuroprotective agent and not just on the apoptotic pathway [54-56]. Moreover, maternal dietary supplementation with

pomegranate juice is neuroprotective for the neonatal brain, leading to markedly decreases in brain tissue loss (60%) in all three brain regions assessed and also to diminish caspase-3 activation by 84% in the hippocampus and 64% in the cortex [57,58].

Resveratrol has been found to be neuroprotective in the adult ischemia model in rats. Treatment with trans-RVT or RVT prevents motor impairment and neurological functions and significantly reduces the volume of infarct and brain edema in middle cerebral artery occlusion (MCAO) model of stroke in rats [58-60]. This natural polyphenol exerts his effect by improving brain energy metabolism (enhancing the level of glucose, ATP and energy charge, and decreasing the levels of lactate) and by attenuating oxidative stress. It inhibits xanthine oxidase activity and prevents the production of hypoxanthine, xanthine and oxygen radicals during ischemia/reperfusion [59]. In addition, it minimizes the generation of ROS, lipid peroxidation (MDA) and NO content and the expression of stress-associated proteins, including heat shock protein 70 (HSP70) and metallothionein [58,60,62]. RVT ameliorates the reduction in the total content of gangliosides, phospholipids, and cholesterol in hippocampus and cerebral cortex [61]. Administrating resveratrol 7 days before global cerebral ischemia, resveratrol brings antioxidant and Na(+)K(+)-ATPase activity in cortex and hippocampus, giving back to normal levels [62] and it remarkably reduces astroglial and microglial activation at 7 days after ischemia/reperfusion (I/R). It is thought that the neuroprotective effect of resveratrol preconditioning may be due in part to the suppression of the inflammatory response via regulation of NF-κB, COX-2 and iNOS induced by I/R [63]. Additionally, histological analysis of CA1 hippocampal region revealed that resveratrol treatment diminishes intercellular and pericellular edema and glial cell infiltration [60, 64], through activation of SIRT1, an NAD+dependent deacetylase. Resveratrol increased expression of SIRT1 and phosphorylation of Akt and p38 but inhibited the increase in phosphorylation of ERK1/2 [64]. Resveratrol increases gene and protein levels of a downstream molecule of SIRT1 (peroxisome proliferator-activated receptor γ coactivator 1α), and mRNA levels of its target genes antioxidative SOD-2 and uncoupling protein 2. It also increases phosphorylation of cyclic AMP-response-element-binding protein and transcription of the anti-apoptotic gene Bcl-2 [62]. Moreover, its effects can be mediated through activation of the PI3-K⁄ Akt signaling pathway and subsequently down-regulating expression of GSK-3beta and

CREB, thereby leading to prevention of neuronal death after brain ischemia in rats [65]. Taking together, these results suggest that the neuroprotective actions of this polyphenol, including anti-oxidative, anti-apoptotic and anti-inflammatory effects, are mediated via modulation of multiple signalling pathways in adult ischemia model in rats.

4.2 DOCOSAHEXAENOIC ACID

Docosahexaenoic acid (22:6n-3) is a long-chain omega-3 fatty acid, commonly found in fish such as salmon and tuna. It has 22 carbons with six double bonds (22:6), and the first double bond is three carbons from the methyl end of the molecule (hence omega-3). DHA is an essential dietary fatty acid because it, or its short chain precursor, alpha-linolenic acid (18:3 n-3), have to be obtained in the diet. In addition, dietary omega-3 fatty acid deficiency is associated with biochemical changes in the brain and with visual disorders [66-70]. In humans, DHA is present in lower concentrations in blood, but in very high concentrations in the brain, retina and spermatozoa. In fact, it is the major polyunsaturated fatty acid in the adult mammalian brain, where constitutes more than 30% of the total phospholipid composition of membrane, being present in three phospholipids (phosphatidylserine, phosphatidylethanolamine and ethanolamine plasmogen). DHA provides plasma membrane fluidity at synaptic regions so it is crucial for maintaining membrane integrity and, consequently, neuronal excitability and synaptic function. As a consequence, DHA is indispensable for maintaining membrane ionic permeability and the function of transmembrane receptors that support synaptic transmission and cognitive abilities [67, 71-73]. Inadequate dietary intakes of omega-3 fatty acids reduce DHA and augment omega-6 fatty acids in the brain.

Decreased DHA in the developing brain leads to deficits in neurogenesis, neurotransmitters metabolism, and altered learning and visual function in animals [74]. DHA accumulates in the brain during late prenatal and early postnatal development; in humans in the third trimester of pregnancy and in rats in the last 3 days of gestation [75]. Clinical trials have proved the importance of feeding term or premature infants with n-3 polyunsaturated acid, and of the maternal intake during pregnancy and

lactation, since the omega-3 are provided during perinatal development through placental transfer and maternal milk, which determines the DHA status of the newborn and consequently impacts on post-natal development of brain and visual functions [74,76].

Docosahexaenoic acid significantly reduces brain volume loss and improves long-term neurological outcomes up to 5 weeks in a neonatal rat model of perinatal hypoxia-ischemia [77-78] and it exerts an anti-inflammatory effect in microglia by inhibiting NF-κB activation and subsequent release of inflammatory mediators [78]. Maternal DHA-enriched diet during pregnancy provides neuroprotection in neonatal brain injury by inhibiting oxidative stress (8-OHdG immunoreactivity were significantly decreased) and apoptotic neuronal death [75]. HI insults interfere with accumulation of brain DHA in developing rats, since 7 days after injury, the ratio of DHA to total fatty acids increases in the control group, but not in the HI group, suggesting that DHA supplementation may be beneficial for treating neonatal HI encephalopathy [78, 79]. In addition, DHA co-treatment with hypothermia produced both sustained functional improvement and reduced brain damage after neonatal hypoxia-ischemia [80].

In the adult model of stroke by MCAO, treatment with DHA significantly improves behavioral disturbance and reduces total infarct volume (by a mean of 40% when administered at 3h, by 66% at 4h and by 59% at 5h), edema and blood-brain barrier disruption [81,82], whereas chronical (but not acute) administration minimizes MDA levels and increases SOD activity after ischemic insult alleviating the oxidative stress in the rat brain [83]. DHA decreases necrosis, mainly modifying membrane biophysical properties and maintaining its integrity in functions between presynaptic and postsynaptic areas, triggering a better stabilization of the intracellular ion balance. DHA also palliates brain apoptosis as well, by inducing antiapoptotic effects; minimizing responses to ROS, upregulating anti-apoptotic and downregulating pro-apoptotic protein expression, and maintaining mitochondrial integrity and function. In animal models DHA renders neuroprotection after HI injury by regulating multiple molecular pathways and gene expression [84]. The protection of neuronal death is associated with increased Nrf2 activation and heme oxygenase-1 (HO-1) upregulation [85]. DHA ameliorates central macrophages/microglia activation, leukocyte infiltration and pro-inflammatory cytokine expression and peripheral leukocyte activation after

cerebral ischemia [80].

Table 1 The mechanisms of action of RVT and DHA in the perinatal hypoxia-ischemia

TARGET in PERINATAL HI	EFFECT of DHA (REFERENCES)	EFFECT of RESVERATROL (REFERENCES)
BRAIN PROTECTION		
Infarct volume/Tissue loss	↓ [77,78]	↓ [52-54,56,58]
Apoptosis	↓ [75]	↓ [54-56]
Morphologically well preserved neurons		↑ [52-54]
White matter injury (MBP/LFB)		↓ [52]
Short-term behavioral deficits		↓ [52-54]
Long-term behavioral deficits (spatial learning and memory)	↓ [77,78,80]	↓ [52-54]
APOPTOSIS		
Caspase-3		↓ [54-56,57]
Bax expression		↓ [54-56]
Bcl-2 expression		↑ [54-56]
Bax/ Bcl-2		↓ [54-55]
Calpain		↓ [56]
ANTIOXIDANT		
DNA oxidation (8-OHdG)	↓ [75]	
INFLAMMATION		
NF-κB	↓ [78]	

5. CONCLUSION

Nowadays there are convincing evidences demonstrating that RVT and DHA treatments are effective against hypoxic-ischemic brain injury in the neonatal rat model, reducing infarct volume and neuronal loss, minimizing lipid and DNA peroxidation, blocking some apoptotic pathways, decreasing inflammation, inhibiting free radical production and increasing the production of some antioxidant enzymes such as GPx and SOD, showed in Figure 2 and Table 1. As hypoxic-ischemic injury is a complex process, an effective therapeutic effect will be obtained only by a strategy that can target multiple pathways. The use of synergic strategies, such as the association between DHA and resveratrol, might lead to a larger neuroprotective effect on the brain thus improving the neonatal outcome. However, there are not bibliographic evidences about the possible synergic pathways of RVT and DHA in hypoxia-ischemia, since it is known that RVT acts via SIRT1 pathways but the effectiveness and precise modes of the neuroprotective action of DHA remain incompletely understood.

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PRETREATMENT WITH RESVERATROL PREVENTS NEURONAL INJURY AND COGNITIVE DEFICITS INDUCED BY PERINATAL HYPOXIA-ISCHEMIA IN RATS

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ABSTRACT

Despite advances in neonatal care, hypoxic-ischemic brain injury is still a serious clinical problem, which is responsible for many cases of perinatal mortality, cerebral palsy, motor impairment and cognitive deficits. Resveratrol, a natural polyphenol with important anti-oxidant and anti-inflammatory properties, is present in grapevines, peanuts and pomegranates. The aim of the present work was to evaluate the possible neuroprotective effect of resveratrol when administered before or immediately after a hypoxic-ischemic brain event in neonatal rats by analyzing brain damage, the mitochondrial status and long-term cognitive impairment. Our results indicate that pretreatment with resveratrol protects against brain damage, reducing infarct volume, preserving myelination and minimizing the astroglial reactive response. Moreover its neuroprotective effect was found to be long lasting, as behavioral outcomes were significantly improved at adulthood. We speculate that one of the mechanisms for this neuroprotection may be related to the maintenance of the mitochondrial inner membrane integrity and potential, and to the reduction of reactive oxygen species. Curiously, none of these protective features was observed when resveratrol was administered immediately after hypoxia-ischemia.

INTRODUCTION

Neonatal hypoxia-ischemia and subsequent brain damage still continue to be an alarming socio-sanitary problem, being considered the single-most important cause of acute mortality and chronic disability in newborns worldwide [1-3]. The result of a deprivation of oxygen and glucose to the brain can lead to death or have severe neurological consequences such as cerebral palsy, mental retardation, visual and hearing impairment, learning and behavioral disabilities, attention deficits, hyperactivity and epilepsy [4-7]. The severity of neonatal encephalopathy depends on the intensity, duration and location of the insult [8,9]. Around 15-20% of affected newborns will die in the postnatal period and an additional 25% will develop severe and permanent neuropsychological sequelae [10]. Only a small percentage of infants with severe injury who survive without any handicap [11,12].

Although theoretically all cell types are affected by hypoxia-ischemia, they do not respond in the same manner. The cells most sensitive to oxygen deprivation are neurons, which exhibit selective vulnerability [13,14], whereas astrocytes are more resistant. Nevertheless, hypoxia-ischemia can prejudice the survival of astrocytes and reduce their capacity for excitatory neurotransmitter uptake and free radical scavenging, which ultimately also influences the survival of neurons [15,16]. Oligodendrocytes, the myelinating cells of the central nervous system, are also particularly vulnerable to hypoxia-ischemia. Damage to these cells leads to myelination deficits, white matter lesions and the destruction of gray matter oligodendrocyte progenitors [17,18].

Hypoxia-ischemia associated brain damage due to impaired glucose and oxygen supply results in immediate neuronal injury and the exhaustion of cellular energy stores, which lead to a multi-faceted cascade of biochemical events. Two distinct metabolic phases have been recognized in the ensuing neurological damage: the first is the primary energy failure due to the hypoxic-ischemic event and the second is a consequence of the re-oxygenation which takes place some hours later [19,20]. Oxidative stress, which is defined as an imbalance between oxidant and antioxidant factors, is considered to be a major contributor to ischemic brain injury [21], because it is an important consequence of the neurotransmitter-mediated toxicity which follows perinatal hypoxia-ischemia. All these changes result in mitochondrial dysfunction and the generation of more injurious reactive oxygen species (ROS), which oxidatively

damage cell constituents, and ultimately lead to neuronal damage or death [22,23]. The human antioxidant defense system does not accelerate in maturation until the late third trimester of pregnancy and as a consequence the human newborn has a relative deficiency in brain superoxide dismutase and glutathione peroxidase [24]. In this sense, it is reasonable to assume that exogenous antioxidant therapy may help to prevent cellular damage when perinatal asphyxia occurs.

Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin produced by different plant species, including grapevines, peanuts and pomegranates [25,26]. The most common dietary source of resveratrol is red wine, and it is believed to be an important factor in the French Paradox, a term which refers to the observation that the French population has a very low incidence of cardiovascular disease, in spite of a diet which is high in saturated fats [27,28]. This polyphenol has been found to play a fundamental role in neuroprotection in models of neurodegeneration such as Alzheimer's, Parkinson's or Huntington's disease [29-32], ischemia and brain and spinal cord injury [33-36]. The neuroprotective effects of resveratrol are due to its antioxidant activity associated with its stilbene structure with two phenol rings. These confer it with the ability to scavenge a variety of free radicals, including lipid peroxyl and carbon-centered radicals, and ROS. In addition, it has also been found to be able to upregulate the expression of several antioxidant enzymes [26,32-37].

Thus the aim of the present work was to characterize the effects of resveratrol administered as a preventive agent (before hypoxic-ischemic injury) or as a therapeutic agent (after hypoxia-ischemia injury) on morphological and cellular damage and on behavioral impairments in hypoxic-ischemic neonatal rats. We chose the Rice-Vannucci rat model to provoke hypoxic-ischemic brain injury because it is a perinatal asphyxia model broadly used to investigate cerebral damage and because the maturity of the rat CNS on postnatal day (P7) is similar to that of human term babies [38,39]. To this end, we evaluated brain damage, focusing on the three principal cell populations of the CNS (neurons, astrocytes and oligodendrocytes), which are the key players in the brain response to insults. Moreover, we analyzed mitochondria and ROS production as these are involved in the cascade triggered after hypoxia-ischemia.Finally, we carried out behavioral studies to evaluate the long-term effects of RVT on cognitive function.

MATERIALS AND METHODS

ANIMALS AND ETHICS STATEMENT

All surgical and experimental procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as in the European Communities directive 2010/63/EU regulating animal research. Experimental protocols were approved by the Committee on the Ethics of Animal Experiments of the University of the Basque Country (UPV/EHU) (Permit Number: CEEA/ 341-344/2014/ALVAREZDIAZ). All surgery was performed under inhaled isoflurane anesthesia, and all efforts were made to minimize suffering.

CEREBRAL HYPOXIA-ISCHEMIA DAMAGE

Neonatal hypoxia-ischemia was induced using the method originally described for immature rats [40] with slight modifications. Thus, seven day-old (P7) Sprague Dawley rats were anesthetized with inhaled isoflurane (3.5% for induction, 1.5% for maintenance) in oxygen, and the left common carotid artery was isolated from nerve and vein and ligated at two locations with 6-0 surgical silk. The common carotid was then transected between the ligatures to guarantee that blood flow through the ipsilateral carotid circulation was absent throughout the experiment. The wound was sutured and the animals were allowed to recover for 2 h in their cages. Once fully recovered from anesthesia, pups were then placed in a humidified container maintained at 36°C. Hypoxia-ischemia was induced by perfusing the container with humidified 8% oxygen in a nitrogen gas mixture with a flow of 5 L/min for 2 h and 15 minutes. After hypoxic exposure, pups were returned to their biological mothers until they were euthanized with sodium pentobarbital (100 mg/kg) injected intraperitoneally at 0 hours, 3 hours and 12 hours after hypoxic-ischemic insult, on postnatal day 14 (P14) or on postnatal day 90 (P90) for experimental studies. If rats showed signs of increased or decreased respiratory rate, decreased activity, loss of appetite, isolation from littermates (indicative of pain or distress in the animal) they were euthanized by trained personnel of the animal unit of the University of the Basque Country (UPV/EHU) using sodium

pentobarbital (in neonates) or carbon dioxide (after weaning). After weaning, animals were housed in individual stalls, and maintained in a climate-controlled environment on a 12-hour light/dark cycle where they had free access to food and water.

EXPERIMENTAL GROUPS

Five experimental groups were established for histological evaluation. Control rat pups were randomly chosen and had neither common carotid artery ligation nor a period of hypoxia (Control, $n \geq 5$). The hypoxic-ischemic group (HI, $n \geq 8$), consisted of animals subjected to hypoxia-ischemia, who received treatment vehicle only [normal saline containing dimethyl sulfoxide (DMSO)]. Resveratrol (Sigma-Aldrich Co. Ltd., Gillingham, UK) was dissolved in DMSO (The Sigma Chemical Co., UK), diluted in normal saline and injected intraperitoneally, as indicated by West et al. (2007) [41]. One group ($n \geq 8$) received a single dose of 20 mg/kg resveratrol 10 minutes before hypoxia (RVT-b) and the other group ($n \geq 7$), immediately after the hypoxic event (RVT-a). Animals that received treatments were randomly assigned among HI groups. A control+vehicle group was established to test the response to the vehicle, but was not found to be different with respect to the control group.

Since we did not observe any morphological signs of neuroprotection with resveratrol administered after ischemia and in order to avoid unnecessary animal experimentation, we decided not to test the efficacy of post-administered resveratrol in flow cytometry and long-term behavioral studies. Thus, for flow cytometry studies, three experimental groups (control, HI and RVT-b) ($n \geq 5$) and three different time points after hypoxic-ischemic insult (0 h, 3 h and 12 h) were evaluated based on the morphological results. Similarly, long-term behavioral studies, that were carried out using P90 control ($n=16$), HI ($n=14$) and RVT-b ($n=10$) rats.

HISTOLOGICAL EVALUATION

TISSUE PROCESSING

Seven days after surgery (on P14), rats were deeply anesthetized with sodium pentobarbital and perfused intracardially with PBS followed by 4% formaldehyde in 0.1 M PBS (pH 7.2-7.4). Brains were removed and immersed in the same fixative solution at 4°C overnight. After dehydration with graded ethanol and xylene, brains were embedded in paraffin wax and cut in 5 µm coronal sections at interaural distance 5.40 mm and bregma -3.60 mm level, according to the Paxinos and Watson atlas [42]. These sections were stained with Nissl (cresyl violet), or immunolabeled with the antibody to myelin basic protein (MBP), to visualize neurons and oligodendrocytes, respectively.

ASSESSMENT OF BRAIN INJURY

To quantify the extent of infarction and to assess the severity of tissue injury, 5 µm paraffin embedded sections were stained with cresyl violet (Sigma-Aldrich Co. Ltd., Gillingham, UK). Photographs of the whole brain from different experimental groups were taken with a Carl Zeiss Stemi 2000-C stereomicroscope. The area of infarction was defined as the area which exhibited loss of the normal cresyl violet staining pattern. Image J software (public domain, National Institutes of Health, http://rsbweb.nih.gov/ij/) was used to measure cross-sectional areas from the rhinal sulcus to the interhemispheric fissure of the left (contralateral side) and right hemispheres (ipsilateral side), based on the intensity and uniformity of the staining. Measurements were performed by a researcher who was blind to the conditions of the treatment. The extent of damage was calculated for both hemispheres as a percentage of ipsilateral damage, using the formula ([C-I]/C)*100, where C is the mean of the contralateral area and I is the mean value of the ipsilateral area for each brain sample.

Brain damage in the different groups was microscopically evaluated by analyzing the CA 1, CA 2-3 and dentate gyrus (DG) of the hippocampus and the parietal cortex (CTX), with an Olympus BX 50 light microscope (x400). Brain injury

was estimated from several cresyl violet histological sections of brains using a semi-quantitative histopathological scoring system, which was a modification of that reported by Hedtjärn et al. (2002) [43]. Evaluations were carried out by a researcher who was blind to the identity of the group being evaluated. Injury in the parietal cortex was graded from 0 to 4 (0= no observable injury; 1= a few small isolated groups of injured cells; 2= several larger groups of injured cells, mild infarction; 3= moderate confluent infarction; 4= extensive confluent infarction encompassing most of the hemisphere). Damage in the hippocampus was assessed in terms of both hypotrophy (shrinkage; score of 0-3) and observable cell injury/infarction (0-3) in each of the three regions studied, resulting in a histopathological score ranging from 0 to 6. Thus, the maximum score for the hippocampus could reach 18 points. Finally, the total score graded from 0 to 28, was the sum score for all four regions and macroscopic evaluations, taking into consideration if the lateral ventricle was symmetric (=0) or asymmetric (=1) and if the ipsilateral one was dilated slightly (=1), moderately (=2) or severely (=3).

REACTIVE ASTROGLIOSIS

In order to evaluate astrogliosis, brains were removed and immersion-fixed in 4% paraformaldehyde and then stored in 30% sucrose until they sank. Coronal sections were cut at a thickness of 60 μm at at interaural distance 5.40 mm and bregma -3.60 mm level. Slices were washed three times in PBS and incubated for 15 minutes in blocking solution (0.25% Triton X-100 in 0.5% BSA in PBS). Sections were incubated overnight at 4°C with a monoclonal rabbit primary antibody to glial fibrillary acid protein (GFAP) (1:1000, Dako, Denmark) diluted in 0.25% Triton X-100 in 0.5% BSA in PBS. The slides were washed three times in PBS and incubated for 1 hour in an anti-rabbit secondary antibody conjugated with Alexa 488 (1:200; Invitrogen) and counterstained with DAPI (1:1000; Invitrogen). Negative controls received the same treatment omitting the primary antibodies and showed no specific staining. Fluorescently immunolabeled sections of whole brain were analyzed using an Olympus Fluoview FV500 Confocal Microscope, taking photos of the CA 1 and dentate gyrus areas of the hippocampus.

ASSESSMENT OF WHITE MATTER INJURY

Slices were dewaxed, rehydrated, washed two times in PBS and treated for 15 minutes with hydrogen peroxide (H_2O_2) (1%) in PBS to inactivate endogenous peroxidases, and then rinsed thoroughly in PBS to completely eliminate H_2O_2. Subsequently, the sections were incubated for 10 minutes in a blocking solution (0.25% Triton X-100 in PBS) and after washed twice in 0.5% BSA in PBS. Sections were incubated overnight at 4°C with a mouse monoclonal primary antibody to MBP (1:100, Santa Cruz Biotechnology, CA, USA) diluted in 0.25% Triton X-100 and 0.5% BSA in PBS. After immunostaining, the slides were washed three times in PBS and incubated for 1 hour with peroxidase-labeled second antibody at a dilution of 1:100 (HRP anti-mouse, Santa Cruz Biotechnology, CA, USA). Finally, the sections were stained with diaminobenzidine, counterstained with hematoxylin (Sigma-Aldrich Co. Ltd., Gillingham, UK), dehydrated in ethanol, cleared in xylene, and coverslipped with DPX mounting medium (Sigma-Aldrich Co. Ltd., Gillingham, UK). Immunolabeled sections of different areas of the brain were analyzed using an Olympus BX 50 light microscope.

White matter integrity was analyzed by measuring the density of MBP immunostaining, using a computerized video-camera-based image-analysis system (Image J software) according to Liu et al. (2002). Evaluations were performed by a researcher who was blind to the identity of the group being evaluated. Unaltered TIFF images were digitized, segmented (using a consistent arbitrary threshold of -50%), and binarized (black versus white). The total number of black pixels per hemisphere was counted, and average values were calculated per brain, and expressed as pixels per hemisphere. Hemisphere areas were also outlined and measured for each section that was analyzed by densitometry. At least three sections per brain were analyzed and only sections with technical artifacts related to the staining procedure were excluded. Densitometric values were expressed as ratios of ipsilateral-to- contralateral hemispheric measurements (I:C).

ASSESSMENT OF MITOCHONDRIAL STATE

TISSUE COLLECTION

For flow cytometry studies, three experimental groups (control, HI and RVT-b) ($n \geq 5$) and three different points of time after hypoxic-ischemic insult (0 h, 3 h and 12 h) were evaluated. After flushing with Ringer lactate solution, the non-fixed tissue samples from fresh ipsilateral brain regions were disaggregated in collagenase (Invitrogen, The Netherlands) solution (1,5 mg/ mL) in Hanks' Balanced Salt solution (HBSS; Sigma-Aldrich, St Louis, Mo, EEUU) at 37 ºC for 20 min and further separated using a cell strainer. Then, cell suspensions were incubated with different fluorochromes and conjugates. Different analyses were performed using an EPICS ELITE Flow Cytometer (Colter, Inc., Florida, USA). To exclude debris and cellular aggregates, samples were gated based on light scattering properties, in side scattering (SSC), which correlates with cell complexity, and forward scattering (FSC), which correlates with cell size, and 10,000 events per sample within a gate (R1) were collected. Events within R1, which corresponded to individual cells, were analyzedfor their fluorescence. An unstained sample was used as a control to determine levels of autofluorescence. Data analysis was performed using the Summit v4.3 software.

Prior to the study by flow cytometry studies, we analyzed the viability in cell suspensions (live cells vs debris) and evaluated cellularity (cell aggregates and individual cells), excluding debris and cell aggregates. Then, when analyzing samples by flow cytometry, we first analyzed unstained samples from each animal, serving as a negative control to correct for autofluorescence. In this sense, we obtained similar values of autofluorescence for all samples, which were used to establish the same fluorescence gating for all samples in order to exclude autofluorescence.

MITOCHONDRIAL INNER MEMBRANE INTEGRITY

The level of cardiolipin was determined by using the fluorochrome Nonyl Acridine Orange (NAO, Invitrogen, The Netherlands). This marker binds to cardiolipin that is located in the mitochondrial internal membrane and is essential for protein functionality and ATP synthesis. 750 µl of cell suspensions ($1x10^6$ cells/mL) were

incubated with 4 µl NAO (10^{-2} M) in PBS at 4 °C and in the dark conditions for 30 minutes and later cells were washed twice in buffer. Samples were immediately evaluated using flow cytometry. Only integral cells were quantified by means of the NAO method, and therefore detritus and/or fragmented cells were not measured.

MITOCHONDRIAL TRANSMEMBRANE POTENTIAL

Mitochondrial transmembrane potential was analyzed using Rhodamine 123 (Rh123, Invitrogen, The Netherlands), a lipophilic cationic fluorochrome, which accumulates inside the mitochondria in proportionto the mitochondrial transmembrane potential. A decrease in the fluorescence of Rh 123 indicates a loss of mitochondrial transmembrane potential. The cell suspension was incubated with Rh123 (4 µl/100 ml) in HBSS for 30 min at 37 °C, followed by washing and incubation in HBSS for 30 min; cells were subsequently washed twice in buffer. Finally samples were analyzed using the flow cytometer.

REACTIVE OXYGEN SPECIES

Intracellular ROS formation was detected using fluorochrome 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Invitrogen, The Netherlands). This probe is cell-permeable and is hydrolyzed intracellularly to the DCFH carboxylate anion that is retained in the cell. Two-electron oxidation of DCFH results in the formation of a fluorescent product, dichlorofluorescein (DCF), which can be monitored by flow cytometry. Cell suspensions were incubated with DCFH-DA fluorochrome (4 µl/100 ml) in HBSS for 30 min at 37 °C and were later washed twice in buffer before loading into the flow cytometer.

BEHAVIORAL IMPAIRMENT EVALUATION

On P90, we evaluated hypoxia-ischemia induced behavioral impairments by using the open field, hole-board, T-maze and novel object recognition tests in control, HI and RVT-b groups. All tests were performed by a researcher who was blind to the group to which the animals pertained.

OPEN FIELD TEST

The open-field consists of a rectangular container made of dark polyethylene (60×60×30 cm) to provide optimal contrast to the white rats in a dimly lit room. The base of the cage is divided by lines in peripheral squares (12) and central squares (4). Testing was conducted in a silent room with constant light (300 lux). Rats were individually placed in the center of the apparatus to initiate a 30 minutes test session. Each session was recorded with a video camera and directly analyzed with the SMART (Spontaneous Motor Activity Recording & Tracking) v.3.0 software system (Panlab, Barcelona, Spain). Total, central and peripheral covered distance (mm), velocity (mm/s) and the time spent in both areas were analyzed.

HOLE-BOARD TEST

The hole-board test was carried out using a grey iron plate covered with dark formica (62×62×36 cm) with a raised floor insert (7 cm above the floor) with 16 holes, each with a diameter of 5 cm. Each rat was placed in the center of the apparatus and left to explore the arena for five minutes (Lee et al., 2014). The frequency of and time spent head dipping into the holes was recorded as a measure of neophobia. A head dip was scored when the head was introduced into the holes at least to the level of the eyes.

T-MAZE TEST

Working memory was tested using a T-maze alternation task. The experiments were performed in a T-maze constructed of wood and painted brown. The walls were 23

cm high, and the alleys were 18 cm wide. The length of the main alley was 31 cm, and the length of the side alleys was 31 cm. The side alleys were closed off from the main alley by movable doors. A week before habituation, all animals were partially food restricted (each female received 15 g of food per day and each male received 20 g of food per day) and remained that way throughout the remaining part of the experiment. This maintained each animal above 85% of its free-feeding body weight. The T-maze was cleaned between different animals but not between different trials. The food reward was a 5 g food pellet. The full experiment consisted of three parts: habituation, training, and testing. During habituation, all animals were placed on the T-maze until they ate two pieces of food or 90 s had elapsed. This was repeated three times a day for five days. During training, all animals underwent six trials a day. Each trial consisted of two runs: a forced run and a free run. On the forced run, rats were forced to obtain a piece of food from one goal arm of the T-maze, with the other goal arm blocked by its door. Animals were then placed back into the start arm for a 10 s delay period. At the beginning of the free run, the rats were allowed to choose either goal arm. If the rats chose the arm opposite the one they had been forced into during the forced run, they received the food reward. If the rats chose the same arm into which they had been forced, they received no food reward. There was a 5 minutes inter-trial interval. The training period ended after control animals made 70% correct choices on two consecutive days. Rats were then tested for their performance at 10 or 40 s delay periods. Rats were given three 10 s delay and three 40 s delay trials during the day of testing. The sequence of delays and forced-run food locations (left or right) were randomized each day, with the stipulation that the same delay or the same forced-arm location could not be used for three trials in a row. Rats were then tested for their performance in the maze recording the number of correct entries. Goal entries were defined as the placing of the four paws in the arm.

NOVEL OBJECT RECOGNITION TEST

This test is a nonrewarded paradigm that measures visual episodic memory. Animals were first habituated to the experimental room for a 30 min period. On the first day, animals were habituated to the apparatus (15x28x50 cm**)** for a 10 min period. On the next two days, animals were allowed to freely explore two identical novel objects

for a 10 min period. On the test day, one of the objects was replaced by a new different one and the animal was allowed to freely explore for 10 minutes. Exploratory behavior was scored for investigation time of each object in the test session. The discrimination index ([time in new object minus time in familiar object] / [time in new object plus time in familiar object]) was defined as the parameter for evaluation.

STATISTICAL ANALYSES

All data were expressed as the mean ± standard error of the mean (SEM), and were analyzed using a one-way analysis of variance followed by Bonferroni-Dunn correction. Statistical analysis was performed using GraphPad Prism version 5 (Graph Pad Software, San Diego, CA, USA).

RESULTS

RESVERATROL PRETREATMENT BUT NOT POST-TREATMENT PROTECTED AGAINST BRAIN INJURY

Representative photographs of coronal sections of the perinatal brains of the different experimental groups are shown in Fig 1. Retraction of the ipsilateral hippocampus and loss of cortical volume as a consequence of the increased ventricular size were characteristics of the HI group in P14 animals. In general these brains revealed an infarct area on the ipsilateral side with loss of brain tissue (Fig 1B), while no macroscopic differences could be observed between control (Fig 1A) and animals treated with resveratrol 10 minutes before hypoxia (Fig 1C). However, animals receiving resveratrol immediately after hypoxia showed an infarct area which was similar to that of the hypoxia-ischemia group (Fig 1D).

A quantitative evaluation of infarct area is shown in Fig 1. The HI group was found to have a high percentage of damage (30.1±2.4%) in comparison to control (0.9±1.2%), while no statistical difference was found between the RVT-b (5.1±0.6%) and control groups ([$F(3,34) = 62.24$, $P < 0.0001$]). No signs of infarct could be observed in resveratrol pretreated animals, but in the RVT-a group, a significant increase in the affected area of the brain (25.1±2.2%) was found, with values similar to those of the HI group. The mortality rate was 10% in the HI group, 8% in the RVT-a group, and 0% in control and RVT-b groups.

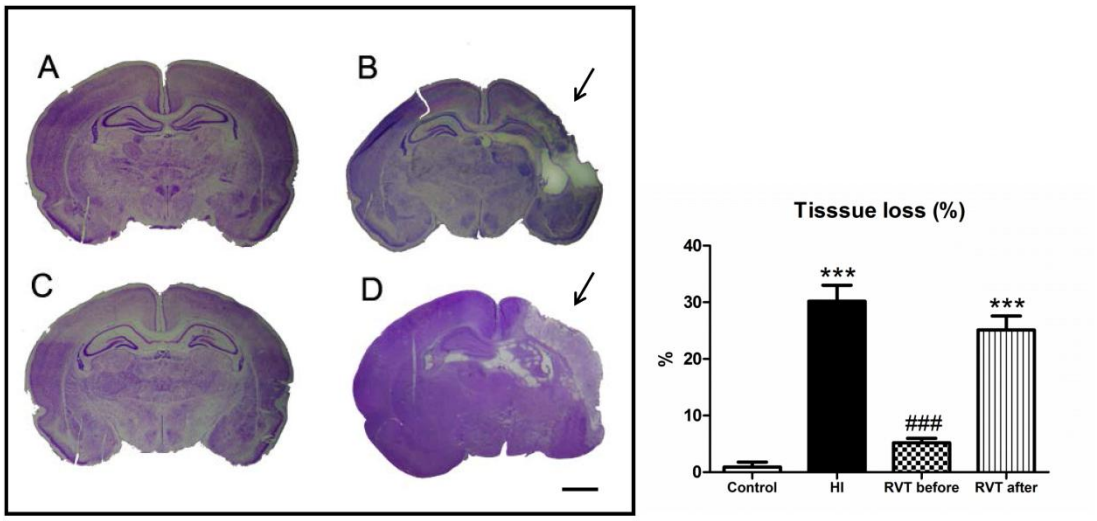

Fig 1. Brain tissue loss induced by hypoxia-ischemia in 7 day-old rats and evaluated at P14. Representative stereomicroscopic photographs of 5 µm Nissl-stained brain sections (interaural distance 5.40 mm and bregma -3.60 mm) are shown. (**A**) Control group with normal morphological brain (n=8). (**B**) HI brain with evident loss of tissue in the ipsilateral side of the cortex, and with obvious damage to the hippocampus (region indicated by arrows) (n=10). (**C**) Brain treated with resveratrol 10 minutes before hypoxia, which is similar to the control brain (n=10). (**D**) HI brain treated with resveratrol immediately after hypoxia, with obvious signs of infarct, as denoted by the arrow (n=10). Scale bar: 2.5 mm. The histogram illustrates percentage of tissue loss is expressed. Asterisks denote the significance levels when compared to the control group (***P<0.0001). The hash denotes the significance levels when compared to the HI group ($^{###}P$<0.0001).

The hippocampus and the parietal cortex of hypoxic-ischemic animals displayed significant evidence of infarction, whereas those of control animals did not. In Nissl stained brain sections, hypoxia-ischemia induced a significant cell loss (see arrows in Fig 2). Moreover, asphyctic animals showed swollen and deformed neurons especially in the ipsilateral CA 1 and CTX areas. In contrast, only mild cell loss and a few damaged neurons were observed in slices in all brain regions studied from animals pretreated with resveratrol, Overall, the subfields in the hippocampus and the parietal cortex were similar in structure to those of the control group. Animals treated with resveratrol immediately after hypoxia presented extensive cell loss, as well as swollen and deformed cells, with overall characteristics which were similar to those exhibited by the hypoxia-ischemia group.

Results from histopathological scoring (Fig 3) corroborate those seen under microscopy (Fig 2). Thus, animals that received resveratrol before hypoxia demonstrated significantly lower values of neuropathology in the CA 1, CA 2-3, DG and CTX, than those of the HI group; CA 1 ([F(3,30) = 35,18, $P < 0.0001$]), CA 2-3 ([F(3,30) = 39.49, $P < 0.0001$]), DG ([F(3,30) = 61.43, $P < 0.0001$]) and CTX ([F(3,30) = 22.75, $P < 0.0001$]). This improvement was more evident in the dentate gyrus and CA 1, with values being similar to those of controls (control group not shown, because its histopathological score for all the areas was 0). In particular, the RVT-b group got significantly lower score in the whole hippocampus (the sum of each hippocampal area) (7.125) in comparison to the same summed area of the HI group (15.13) and RVT-a group (17). The overall, total score was also significantly ($p<0.0001$) reduced in the RVT-b group, with 9.25 points versus the 22.25 and 25 points of the HI and RVT-a groups, respectively. In contrast, when resveratrol was administered immediately after the injury, no neuroprotective effects in terms of an improved histopathological score. This assessment of the brain injury indicates that resveratrol pretreatment but not post-treatment reduced infarct volume and attenuated cell damage.

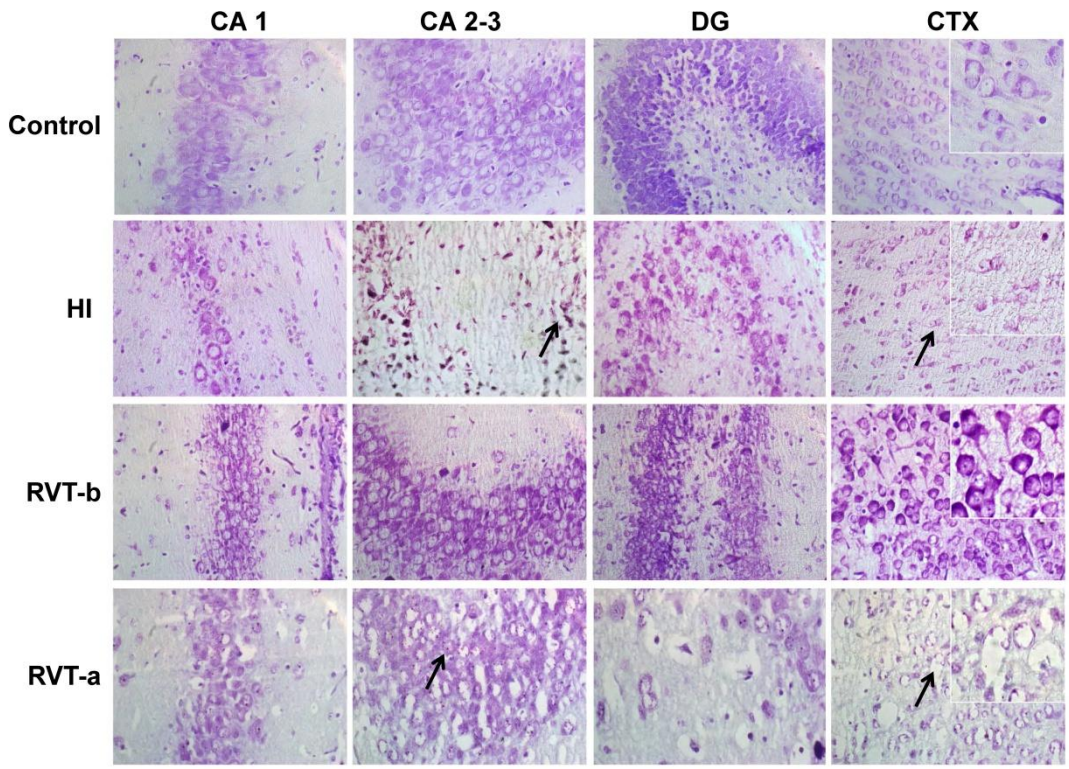

Fig 2. Representative microphotographs of Nissl-stained brain sections in animals exposed to hypoxia-ischemia at P7 and evaluated at P14. Individual fields represent different experimental groups and different areas of the hippocampus (CA 1, CA 2-3 and DG) and from the parietal cortex (CTX), with high magnification insets in CTX. Cell loss was especially evident in the DG and parietal cortex (arrows) in the hypoxia-ischemia and resveratrol post-treatment groups. In contrast, the resveratrol pre-treated group showed a remarkable conservation in the cellularity of the different studied areas with respect to the HI group. Scale bar: 50 µm.

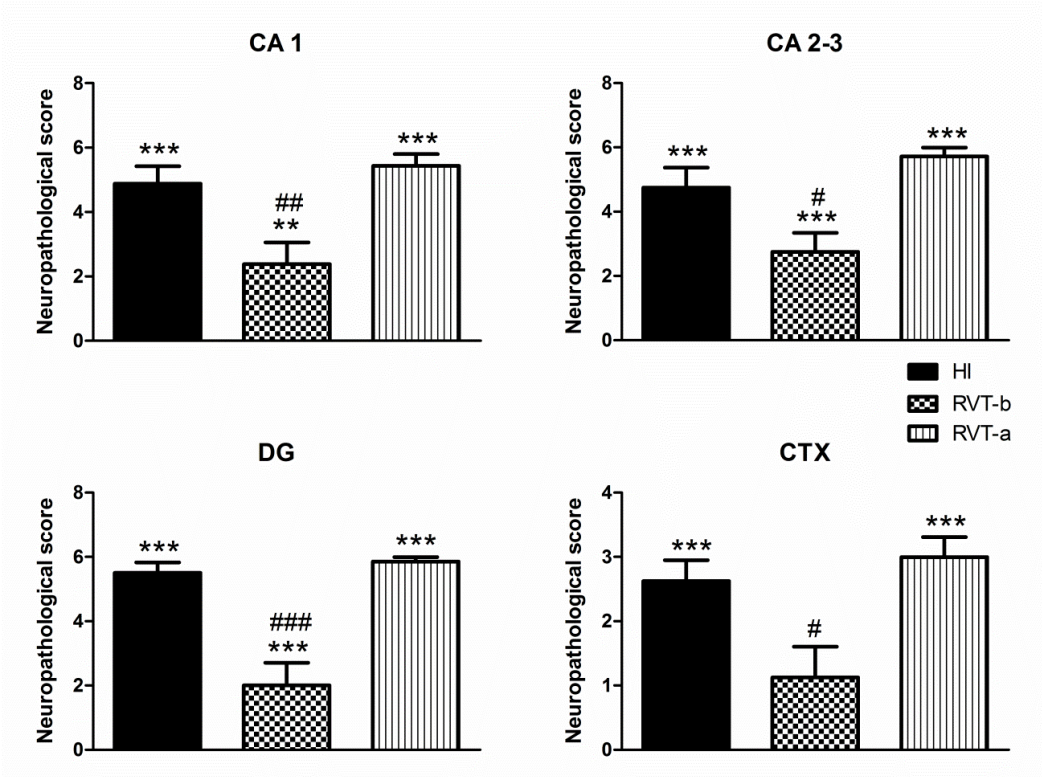

Fig 3. Histopathological score of damage in P14 rat brains of different groups expressed as the mean ± SEM. Asterisks denote the significance levels when compared to the control group (***P<0.0001). The control group (n=11) is not shown, because it histopathological score for all areas was 0. The hash symbols denote the significance levels when compared to the HI group ($^{###}P$<0.0001). It can be clearly seen that the group pretreated with resveratrol (n=8) had a lower histopathological score compared with the HI group (n=8). This was clearly not the case for the post-resveratrol treated group (n=8).

PRETREATMENT WITH RESVERATROL MINIMIZED THE ASTROGLIAL REACTIVE RESPONSE

GFAP astrogliosis was found in animals with hypoxic-ischemic injury, particularly in regions near dead or dying cells, such as the CA1 and dentate gyrus areas of the hippocampus, while control cases showed low levels of GFAP immunoreactivity (Fig 4). This reactive astrocyte response was diminished when resveratrol was administered before hypoxia, but not after it, demonstrating that pretreatment with resveratrol reduced the astroglial reactive response.

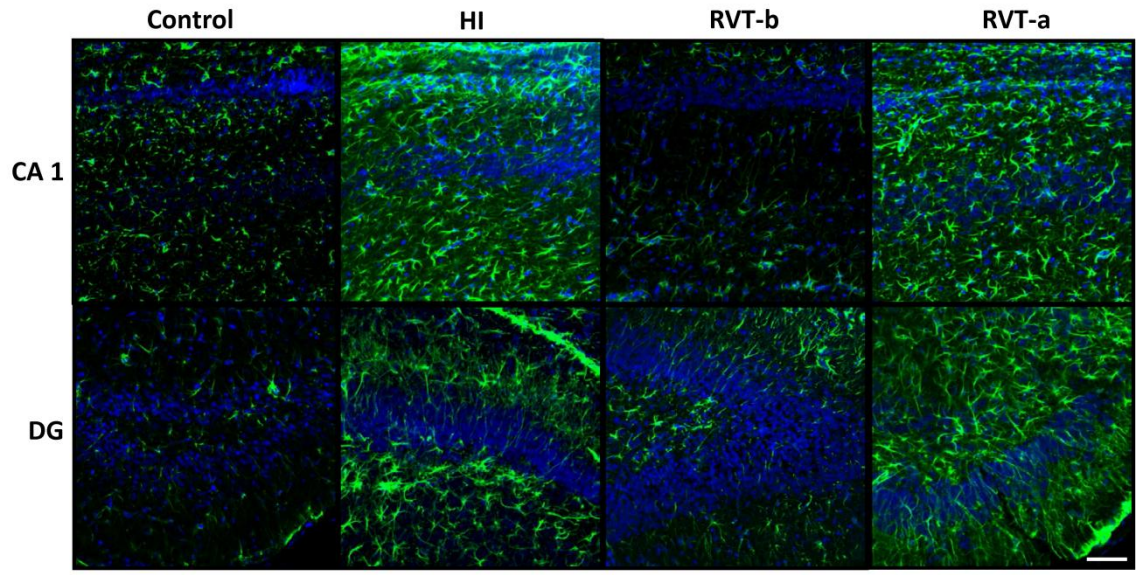

Fig 4. Representative confocal microphotographs of glial fibrillary acidic protein (GFAP)-immunoreactivity in brain sections counterstained with DAPI. On postnatal day 14, GFAP immunoreactivity (green) was particularly pronounced in the vicinity of damaged areas and this reactivity was substantially reduced in animals pre-treated with resveratrol. Scale bar: 40 μm.

RESVERATROL PRETREATMENT PRESERVED MYELINATION

A substantial loss of ipsilateral MBP immunostaining was observed in both external capsule and striatum in P14 animals that had been subjected one week earlier to hypoxia-ischemia with respect to controls (Figs 5 and 6). This loss was absent with resveratrol pretreatment, but not when resveratrol was administered after the injury.

Quantitative analysis corroborated these initial impressions from microscopy observation. At the level of the external capsule (Fig 5), a substantial loss of ipsilateral MBP immunostaining ([$F(3,36) = 11.63$, $P < 0.0001$]) was observed in the HI group (0.86) when compared with control animals (0.99). Pups pretreated with resveratrol showed a smaller degree of MBP loss in the ipsilateral hemisphere (0.98), whereas this

improvement was not apparent in the RVT-a group (0.83) whose MBP (I:C) ratio was similar to that observed in the HI group.

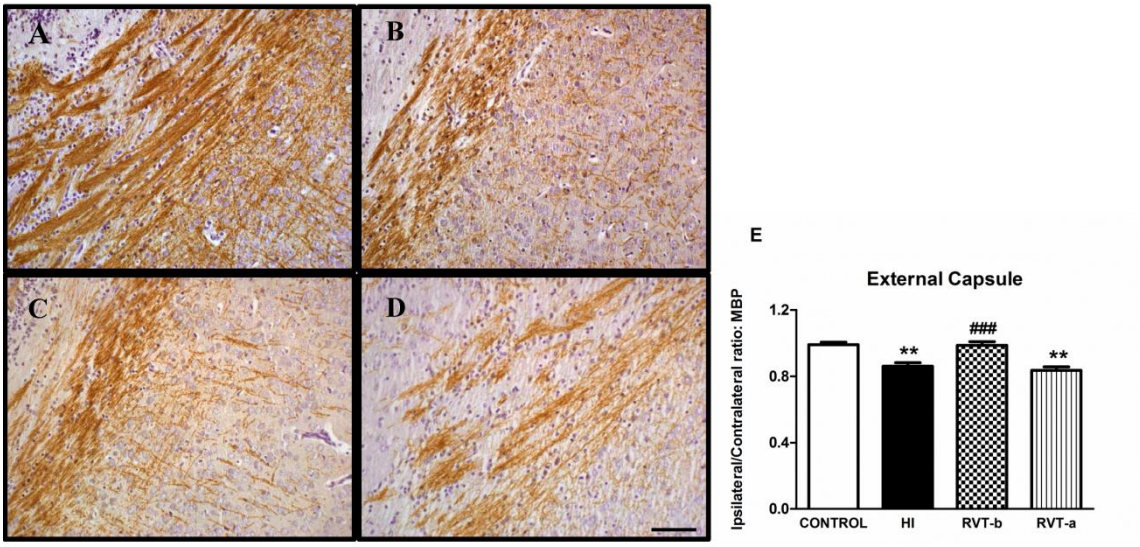

Fig 5. Representative light microphotographs of myelin basic protein (MBP)-stained brain sections and comparison of loss of MBP immunostaining in the external capsule of different groups: 14-day-old control (n=5) (**A**), HI (n=14) (**B**), RVT-b (n=14) (**C**) and RVT-a (n=7) (**D**) (scale bar: 40 μm). In the histogram (**E**), the extent of tissue injury, expressed as a ratio of left-to-right hemispheric MBP immunostaining is represented. Asterisks denote the significance levels when compared to the control group (**P<0.0001). Hashes denote the significance level when compared to the HI group ($^{###}P$<0.0001).

At the level of the mid-striatum (Fig 6), HI neonatal rats (0.68) exhibited a significant ([F(3,37) = 48.49, P < 0.0001]) loss in MBP immunostaining in subcortical white matter when compared with control animals (1.01). Resveratrol-pretreated animals showed a lesser degree of MBP loss in the ipsilateral hemisphere (1.01), with a similar MBP ratio (I:C) to that observed in the control group. Additionally, there was also a significant reduction in the I:C immunostaining ratio in animals who received

resveratrol after injury (0.68). These results suggest that resveratrol when administered as a preventive agent is able to ameliorate the loss of myelination.

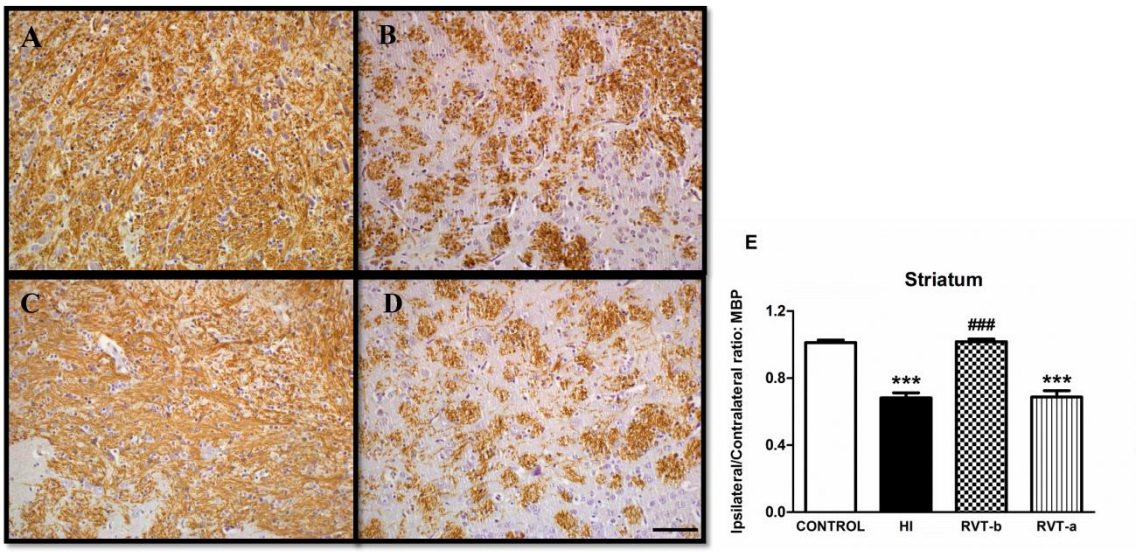

Fig 6. Representative light microphotographs of myelin basic protein (MBP)-stained brain sections and comparison of loss of MBP immunostaining in the striatum of different groups: 14-day-old control (n=6) (**A**), HI (n=15) (**B**), RVT-b (n=13) (**C**) and RVT-a (n=7) (**D**) (scale bar: 40 μm). The histogram (**E**) illustrates the extent of tissue injury associated with the various treatments and values are expressed as a ratio of left-to-right hemispheric MBP immunoreactivity levels. Asterisks denote the significance levels when compared to the control group (***P<0.0001). Hashes denote the significance levels when compared to the HI group ($^{###}P$<0.0001).

ASSESSMENT OF MITOCHONDRIAL STATE

MITOCHONDRIAL INNER MEMBRANE INTEGRITY WAS PROTECTED BY RESVERATROL

Integrity of the inner mitochondrial membrane was evaluated using the fluorochrome NAO. The initial states, 0 h and 3 h, showed no statistically significant differences among all groups (Figs 7A and B). However, at 12 h ([$F(2,20) = 6.006$, $P < 0.05$]), the percentage of NAO positive cells significantly decreased in the HI group (76.53±10.71%) with respect to the control group (97.37±0.61%), suggesting reduced mitochondrial integrity, while animals pretreated with resveratrol maintained mitochondrial inner membrane integrity (99.42±0.06%), and showed similar values in comparison to the control group (Fig 7C).

Nevertheless, we found that at initial time, 0 h ([$F(2,21) = 5.024$, $P < 0.05$]), the HI group underwent a diminishment with statistical differences in the relative values of fluorescence intensity for NAO (55.52±5.39%) with respect to the control group, but the RVT-b group did not show a significant reduction(81.65±13.55%) (Fig 7D). At 3 h there was no significant difference among the groups (Fig 7E). In contrast, at 12 h ([$F(2,15) = 8.06$, $P < 0.05$]) after resveratrol administration the relative values of NAO fluorescence intensity were significantly higher (193.2±17.93%) than in the HI group (62.6±3.69%) and even in the control group (Fig 7F). These data indicate that resveratrol protected the integrity of the mitochondrial inner membrane.

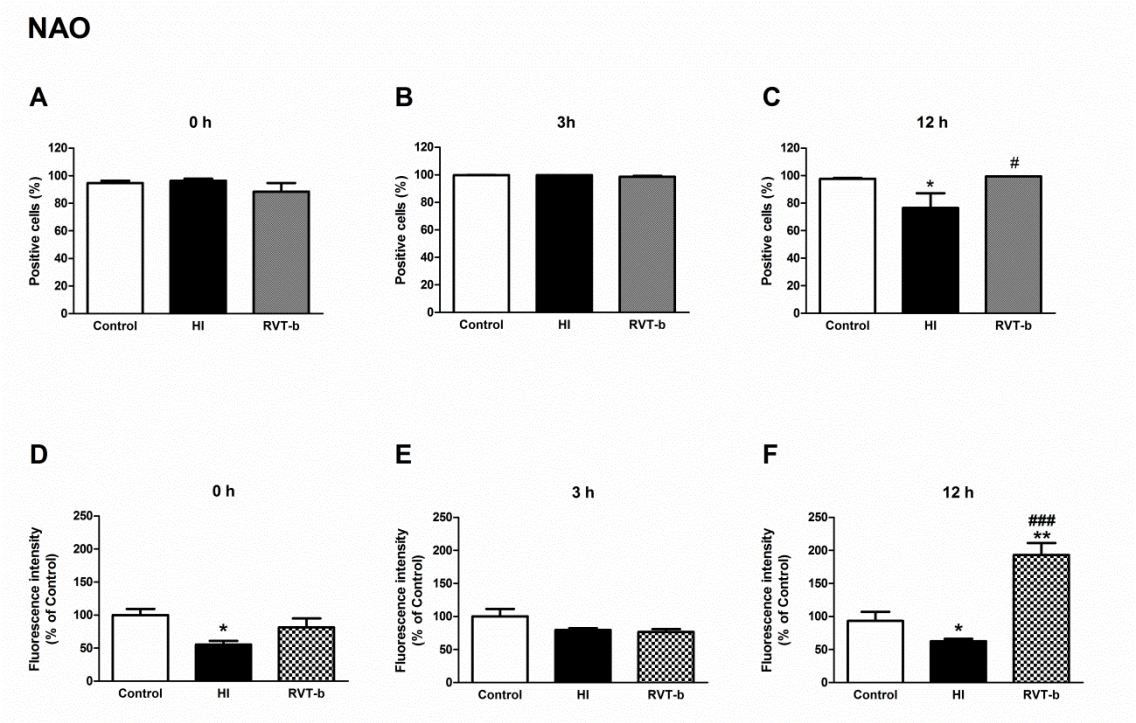

Fig 7. Mitochondrial inner membrane integrity evaluation in suspension of acutely isolated cells using nonyl acridine orange (NAO). Percentage of NAO-positive cells at different time points after hypoxia-ischemia: (**A) 0 h, (B) 3 h and (C) 12 h. Relative fluorescence intensity of cells with *in vivo* marker NAO at different time points after hypoxia-ischemia**: **(D) 0 h, (E) 3 h and (F) 12 h**, in control (n≥5), HI (n≥5) and animals pretreated with resveratrol (n≥5). Asterisk denotes the significance levels when compared to the control group (*****P<0.05). The hash symbol denotes the significance levels when compared to the HI group ($^{\#}P$<0.05).

EVALUATION OF MITOCHONDRIAL TRANSMEMBRANE POTENTIAL

Mitochondrial transmembrane potential was analyzed using the fluorochrome Rhodamine 123. At 0 h ([F(2,19) = 10.8, P < 0.005]), hypoxia-ischemia generated a decrease in the percentage of Rh 123 positive cells (82.89±3.78%), in comparison to the control group (98.14±0.47%). In contrast, the RVT-b group showed a percentage of Rh 123 positive cells (93.25±1.18%) which was similar to that of the control group (Fig 8A). At 3 h ([F(2,12) = 6.84, P < 0.05]), the number of Rh 123 positive cells diminished in HI animals (91.99±2.28), and also in the resveratrol pretreated group (92.91±1.71%). Although these reductions were statistically significant, they were not as evident as the differences at 0 h with respect to control (99.88±0.03%) (Fig 8B). However, at 12 h

([F(2,24) = 28.02, $P < 0.0001$]) animals subjected to the hypoxic-ischemic event underwent again an important diminishment in the percentage of Rh 123 positive cells (76.08±3.91%) in comparison to the control group (98.81±0.37%), while animals pretreated with resveratrol showed similar values (90.39±1.59%) to those of the controls (Fig 8C).

At 0 h ([F(2,21) = 5.088, $P < 0.05$]), the relative values of Rh123 fluorescence intensity in cells isolated from HI rats were reduced (55.13±5.44%), whereas in those from animals pretreated with resveratrol, the observed values (81.75±13.34%) were found to be similar to those of the control group (Fig 8D). In contrast, at 3 h ([F(2,12) = 4.174, $P < 0.05$]) there was not a significant difference between control and HI groups, but animals that received resveratrol exhibited higher values (115.2±13.34%) than the HI group (89.2±6.52%) (Fig 8E). At 12 h there was no significant difference among the groups (Fig 8F). Representative fluorograms of mitochondrial transmembrane potential measured ash Rh 123 fluorescence also revealed that HI animals had lower values of fluorescence than control and treated animals (Fig 9). These results provide evidencethat resveratrol maintainsd mitochondrial transmembrane potential.

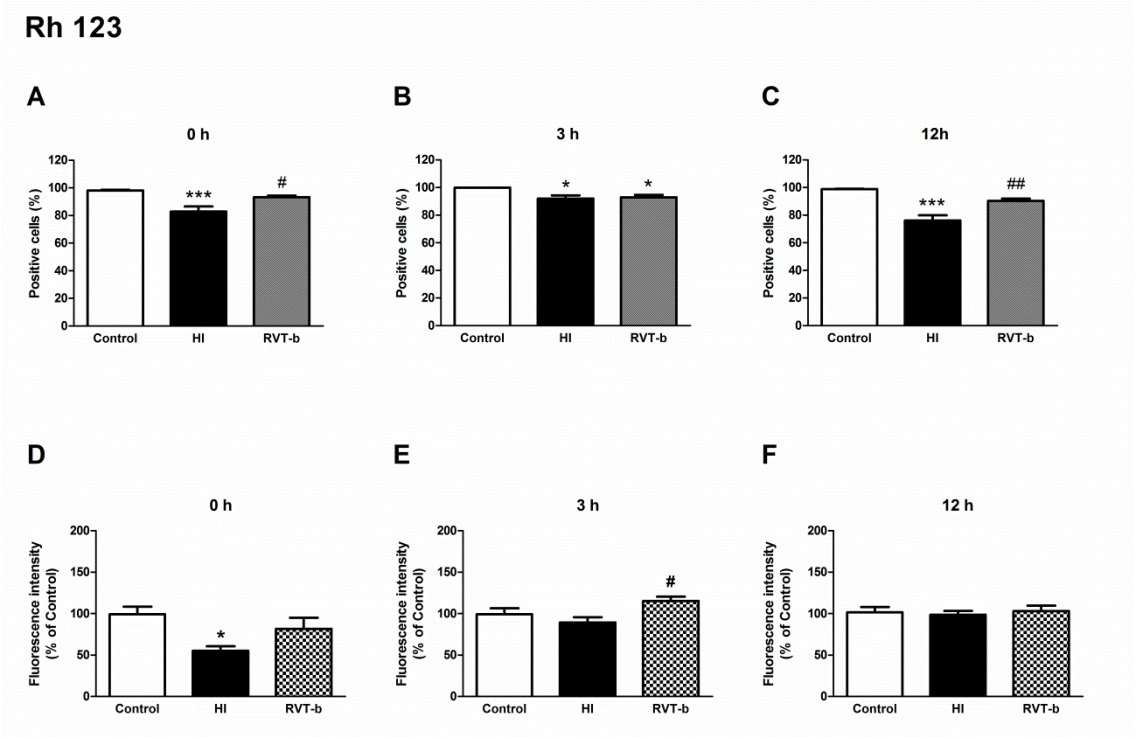

Fig 8. Analysis of mitochondrial transmembrane potential by Rhodamine 123 in suspension of acutely isolated cells. Percentage of cells labeled with the *in vivo* marker Rh 123 at different time points after hypoxia-ischemia: (**A**) **0 h, (B) 3 h and (C) 12 h. Relative fluorescence intensity of cells exhibiting Rh 123 fluorescence at different time points after hypoxia-ischemia**: (**D) 0 h, (E) 3 h and (F) 12 h,** in control (n≥5), HI (n≥5) and animals pretreated with resveratrol (n≥5) groups. Asterisks denote the significance levels when compared to the control group (***P<0.0001 or *P<0.05). The hash symbols denote the significance levels when compared to the HI group ($^{\#\#}P$<0.005 or $^{\#}P$<0.05).

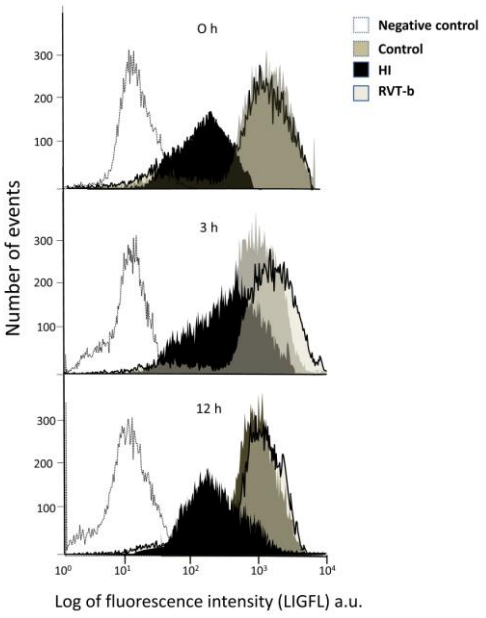

Fig 9. Representative fluorograms obtained after flow cytometry analysis showing mitochondrial transmembrane potential measured as Rh 123 fluorescence at different points of time after hypoxia-ischemia (0 h, 3 h and 12 h) in control, HI and animals pretreated with resveratrol. The x-axis represents the number of events and the y-axis represents the values of fluorescence intensity in logarithm values. The negative control consisted of unstained samples from each animal to remove the autofluorescence.

RESVERATROL REDUCED THE PRODUCTION OF OXYGEN REACTIVE SPECIES

Oxygen reactive species were detected using the DCFH-DA fluorochrome. At 0 h ([F(2,18) = 216,9, P < 0.0001]) while the HI group showed a decrease in the percentage of DCFH positive cells, the treated group presented similar percentage values which were statistically similar to those of the control group (Fig 10A). At 3 h and 12 h, no statistically significant differences were found among all the groups (Fig 10 B,C).

Concerning the relative values of fluorescence intensity indicative of ROS production, at 0 h no significant difference were found among all the groups (Fig 10D). At 3 h ([F(2,13) = 12.84, *P* < 0.0001]) there was an increase in HI rats (197.19±14.93%) and also in RVT-b animals (159.35±15.64%), although this was not statistically significant (Fig. 10E). At 12 h ([F(2,18) = 67.58, *P* < 0.0001]) the relative values of fluorescence intensity of DCFH were significantly increased in the HI (303.7±8.58%) and RVT-b (240.422±28.73%) groups with respect to the control group, but this increase was less pronounced in the RVT-b group (Fig 10F). These results provide evidence that resveratrol reduced ROS production in living cells.

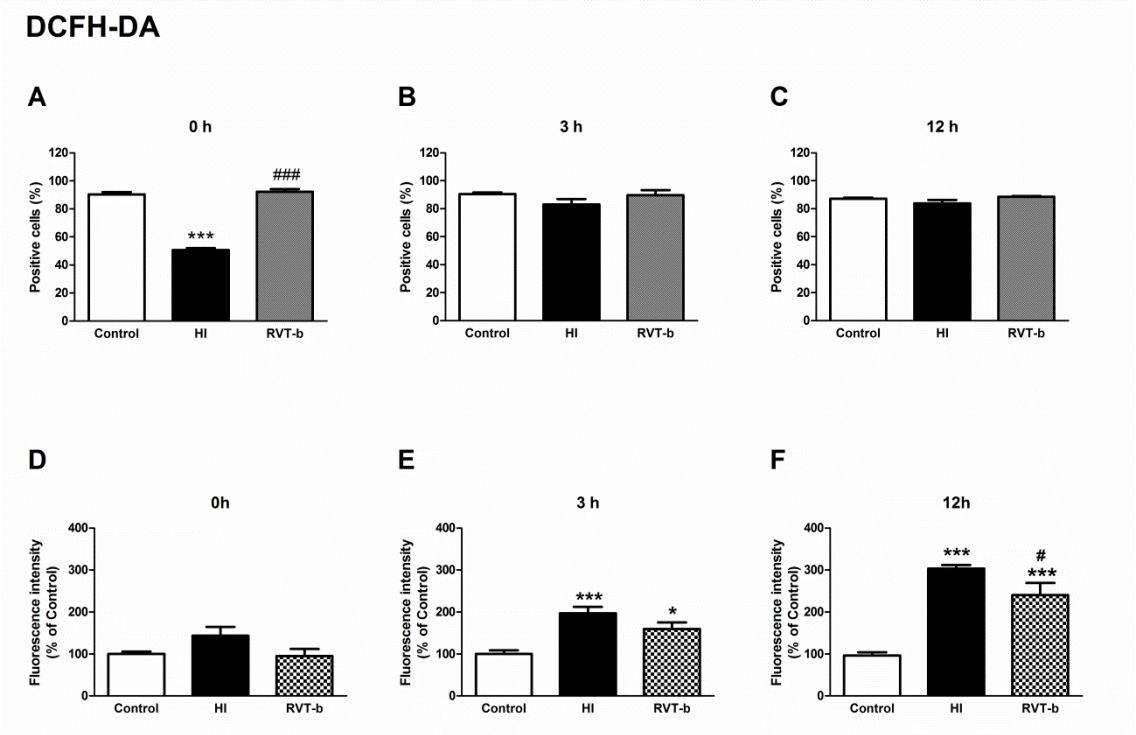

Fig 10. **Effect of brain hypoxia-ischemia on the production of reactive oxygen species in suspension of acutely isolated cells, measured using DCFH-DA. Percentage of DCFH-DA positive cells at different time points after hypoxia-ischemia**: (**A) 0 h, (B) 3 h and (C) 12 h. Relative fluorescence intensity of cells with *in vivo* marker DCFH-DA at different time points after hypoxia-ischemia**; (**D) 0 h, (E) 3 h and (F) 12 h**, in control (n≥5), HI (n≥5) and animals pretreated with resveratrol (n≥5) groups. Asterisks denote the significance levels when compared to the control group (********P*<0.0001 ******P*<0.05). The hash symbols denote the significance levels when compared to the hypoxia-ischemia group ($^{\#\#\#}P$<0.0001 or $^{\#}P$<0.05).

EVALUATION OF BEHAVIORAL IMPAIRMENT

Behavioral deficits were evaluated on day P90 in HI and control animals. Afterwards, we tested if administration of resveratrol 10 min before hypoxia could improve behavioral impairments.

HYPOXIC-ISCHEMIC INJURY DID NOT ALTER MOTOR ACTIVITY

No significant differences were found between control, HI and RVT-b (20 mg/kg) groups in the percentage of time spent in the periphery (Fig 11A) or in the center of the apparatus (Fig 11B). In the same way, no changes were observed in the total distance travelled (mm) (Fig 11C) or speed (m/s) (Fig 11D), demonstrating that hypoxic-ischemic injury did not alter motor activity.

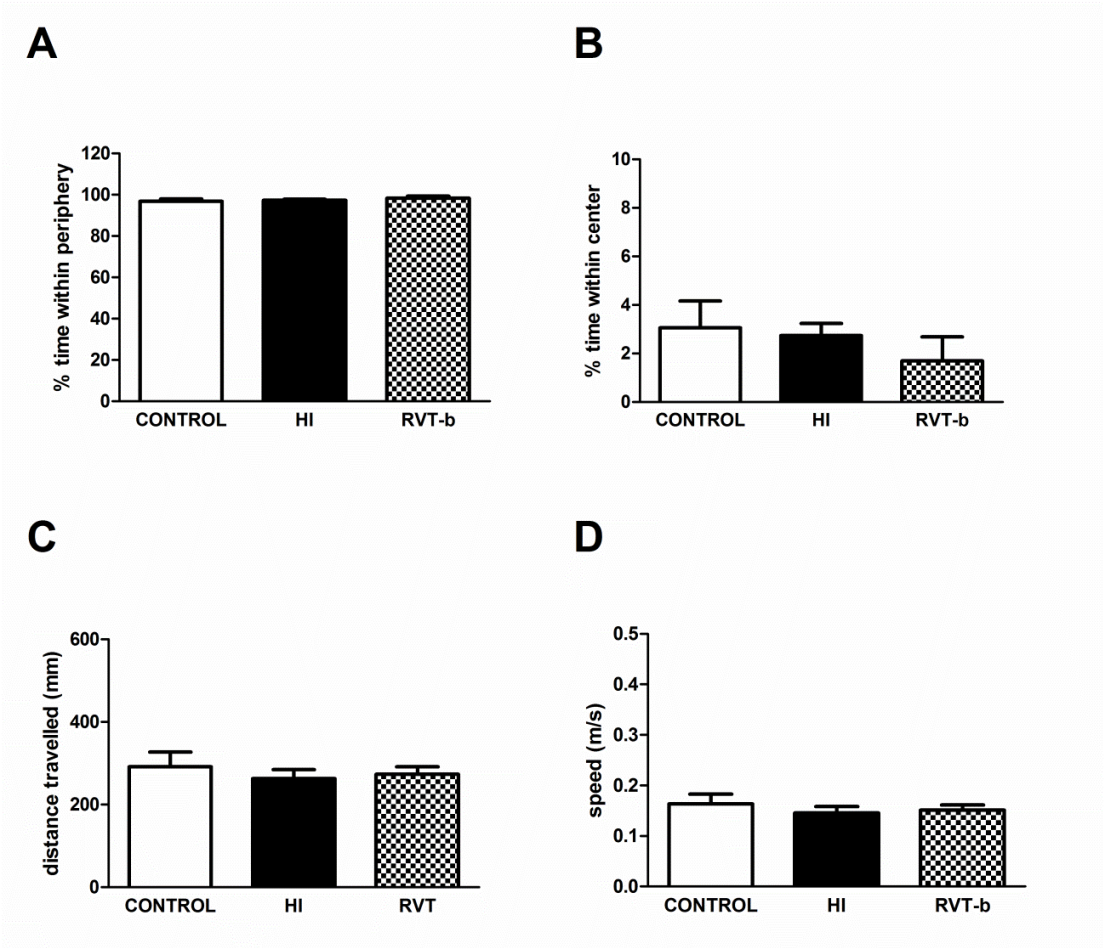

Fig 11. Evaluation of spontaneous locomotor activity in the open field test performed at P90 in control (n=16), HI (n=14) and RVT (20 mg/kg) treated animals (n=10). Evaluated parameters were (**A**) % time in the periphery, (**B**) % time in the center, (**C**) total distance travelled and (**D**) speed in the open field.

RESVERATROL REDUCED ANXIETY AND NEOPHOBIA INDUCED BY HYPOXIA-ISCHEMIA

Rats that underwent hypoxia-ischemia showed a statistically significant increase in the frequency of head-dipping behavior ([F(2,37) = 9.114, $P < 0.005$], when compared to control animals (Fig 12A). In contrast, RVT (20 mg/kg) pretreated animals presented similar values to non-ischemic, control rats, suggesting that RVT is able to reduce the anxiety and neophobia associated with hypoxic-ischemic injury. No differences were found between groups in the hole-board test (HBT) for exploratory time (Fig 12B).

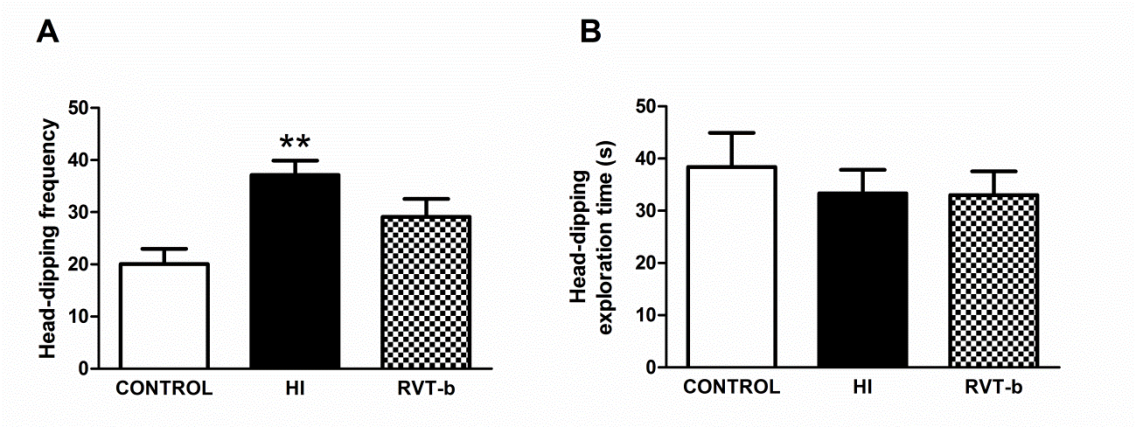

Fig 12. Effect of neonatal hypoxia-ischemia and resveratrol pretreatment on the hole-board test performed at P90. (**A**) The frequency and (**B**) the time spent head-dipping into the holes were recorded in control (n=16), HI (n=14) and RVT (20 mg/kg) treated animals (n=10). Asterisks denote the significance levels when compared to the control group (**$P < 0.005$).

RESVERATROL PRETREATMENT IMPROVED SPATIAL WORKING MEMORY IMPAIRMENTS

At a 10 s delay interval (Fig 13A), no differences were found between groups. In contrast, at a 40 s delay interval ([$F(2,37) = 32.57$, $P < 0.0001$]), HI animals made significantly fewer correct choices (Fig 13B) when compared to control animals. RVT reversed these changes and significantly increased the number of correct choices when compared to HI rats. The results indicate a working memory dysfunction induced by hypoxia-ischemia which is prevented by the acute administration of RVT before the ischemic event.

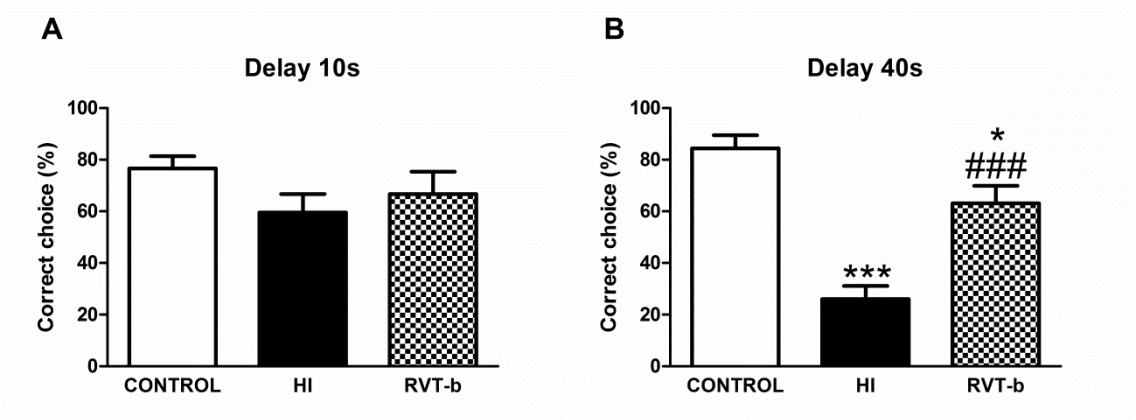

Fig 13. Effect of neonatal hypoxia-ischemia and pretreatment with resveratrol on choice accuracy in the discrete-trial delayed spatial alternation task (T-maze) in adult animals on P90. (**A**) Control (n=16) and HI rats (n=14), as well as RVT pretreated animals (n=10) made a similar number of correct choices in the T-maze at 10 s delay. (**B**) In contrast, HI animals made significantly fewer correct choices after the 40 s delay. Impaired memory performance (percentage of correct trials) due to hypoxia-ischemia was reverted by RVT pre-administration. Asterisks denote the significance levels when compared to the control group (*******P<0.0001). The hash symbols denote the significance levels when compared to the HI group ($^{###}P$<0.0001).

NON-SPATIAL WORKING MEMORY IMPAIRMENT WAS PREVENTED BY RESVERATROL

Hypoxia-ischemia induced a significantly profound decrease in the discrimination index when compared to control animals (Fig 14) ([F(2,38) = 5.106, P < 0.05]). Acute resveratrol fully reverted the effects of hypoxia-ischemia on novel object recognition, recovering discrimination index to control values. Thus, resveratrol appears to prevent non-spatial working memory deficits induced by hypoxia-ischemia.

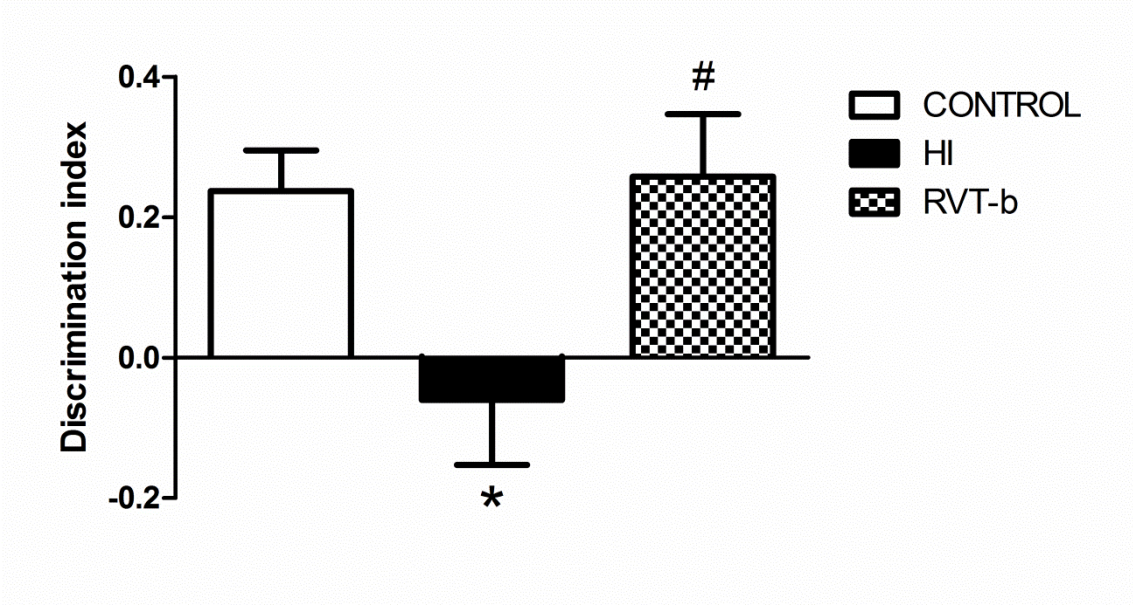

Fig 14. Effect of neonatal hypoxia-ischemia and treatment with resveratrol on the results of a novel object recognition test. On P90, HI adult animals (n=14) displayed a decrease in discrimination index when compared to control animals (n=16) that was fully reversed by resveratrol pretreatment (n=10). Asterisk denotes the significance levels when compared to the control group (*****P<0.05). The hash symbol denotes the significance levels when compared to the HI group ($^{\#}P$<0.05).

DISCUSSION

The aim of the present work was to characterize the effects of resveratrol administered either as a preventive agent or as a therapeutic agent in hypoxic-ischemic brain injury in neonatal rats.We have found that pretreatment with resveratrol,but not post-treatment, protected the brain from subsequent hypoxic-ischemic damage in different ways and that the neuroprotective effect of this polyphenol was long lasting, since behavioral outcomes were significantly improved in pre-treated ischemic animals when assessed at adulthood.

Resveratrol, when administered before the hypoxic-ischemic event, was found to be a potent protective agent that diminished tissue loss and consequently the infarct area, as reported byWest et al. (2007) [41] and Karalis et al. (2012) [44]. Concerning astrocytes, reduced glial reactivity in the resveratrol pre-treated group suggests that the magnitude of the impact of the ischemic stress had been reduced. In this sense, although partial astrogliosis can confer neuroprotection by scavenging ROS and assisting with reconstruction following brain injury [45], excessive astrogliosis can result in the impairment of neuronal signaling and the disruption of myelination [46,47]. White matter damage and late cognitive impairment are important deleterious effects known to occur after hypoxic-ischemic injury in preterm children [448]. A widespread and complex inflammatory response to ischemia occurs which finally contributes to white matter injury [49], principally via the jeopardizing of oligodendroglia, since these cells are particularly susceptible to glutamate-mediated injury. The loss of MBP was absent when resveratrol was given before the injury, thus corroborating the findings of Karalis et al. (2011) [44].

Using immunohistological techniques, we did not observe any signs of neuroprotection with resveratrol administered after hypoxia-ischemia, in keeping with the findings of West et al. (2007) [41]. Consequently, and in order to avoid unnecessary animal testing, we decided not to test the efficacy of post-administered resveratrol in flow cytometry and long-lasting behavioral studies. Paradoxically, a protective effect of resveratrol given after hypoxic-ischemic injury has been reported by Karalis et al. (2011) [44]. However, this discrepancy is likely due to their using a shorter period (one hour) and to their use of higher doses of trans-resveratrol (90 mg/kg), since the neuroprotective effects of this stylbene are dose-dependent in this animal model

[41,50,51] Thus, it is possible that resveratrol post-treatments can protect against mild hypoxic-ischemic injury, but not against severe or moderate injury. Similarly, when we administered resveratrol as a therapeutic agent, the damage produced by the deleterious hypoxia-ischemia cascade had already started and the dose of resveratrol which we used was unable to revert the initiated damage

Different studies have demonstrated the neuroprotective actions of resveratrol, including its anti-oxidative, anti-apoptotic and anti-inflammatory effects, in a variety of ischemia models [52-54]. These beneficial effects are known to be mediated via modulation of multiple signaling pathways [55,56]. Mitochondria play a fundamental role in the cascade triggered after hypoxic-ischemic injury, as mitochondrial respiration is suppressed [57]. In the developing brain, lack of oxygen results in a depletion of cellular energy reserves, which trigger several pathophysiological responses, but there is a common convergence at the level of the mitochondria [58]. Our results indicate that in the specific cell population which we isolated, resveratrol maintains a constant number of cells with an intact mitochondrial inner membrane and that those cells presented a high quantity of cardiolipin 12 hours after the injury. In the same way, when resveratrol was administered as a pretreatment the percentage of cells with intact transmembrane potential and the fluorescence intensity was similar to controls, whereas HI animals showed reduced values. This indicates that the cells that are alive maintain their transmembrane potential; in fact, rhodamine 123 accumulates specifically in the mitochondria of living cells [62].

As we have mentioned, oxidative stress is considered to be a major contributor to hypoxic-ischemic brain injury, and our data show that although there were fewer cells producing ROS in the HI group, those which were producing these species were doing so more vigorously This might be partially due to the fact that mitochondria are both a source and a target of ROS [23,63]; after the initial mitochondrial, an increase in the quantity of ROS production was observed to occur overtime. In contrast, this did not happen in the case of resveratrol pretreatment which ameliorates the production, as a result of its antioxidant effect. In addition to reducing the quantity of ROS production, we speculate that one of the ways resveratrol might protect against hypoxic-ischemic damage may be by protecting mitochondrial integrity and maintaining the membrane potential. Previous findings have demonstrated that resveratrol exerts one of its neuroprotective effects in this way [23], and also by modulating mitochondria [56],

inducing mitochondrial biogenesis against brain ischemic stroke [64] and preserving brain mitochondria functions after hypoxia-reoxygenation [50].

One of the most significant findings of this study is the evidence that resveratrol when administered before hypoxia-ischemia, was able to improve the long-lasting cognitive deficits induced by hypoxia-ischemia, probably via the protection and preservation of neocortical and subcortical brain areas (sensorimotor cortex, hippocampus and striatum), and in particular, of neuronal networks responsible for learning and memory. The beneficialeffects of resveratrol on cognition have been reported in other pathologies in rats such as epilepsy [67] a stress model [68] and in a prenatal stress model [69]. More importantly, this polyphenol has been shown to increase memory performance in primates and to increase hippocampal functional connectivity in older adults [70], in addition to playing an important role in enhancing cerebrovascular and cognitive functions in humans [71].

Hypoxia-ischemia did not seem to exert any obvious effects on basic motor abilities, corroborating the findings reported by Damoradan et al. (2014) [73]. This may partly be due to the high degree of cerebral plasticity of rats. Compensatory reorganization of vital functions following injury is possible and the contralateral hemisphere can functionally take over certain tasks from the injured hemisphere [74,75]. Anxiety-like behaviors observed in rats that underwent hypoxia-ischemia [76,77] were prevented by resveratrol. Head-dipping behavior in the HBT is considered to be a good index for evaluating the anxiety of rodents [78]. Impairments in reference and working memory evaluated in the T-maze performance were demonstrated after neonatal hypoxia-ischemia, as previously reported [72,75,79-83]. However, resveratrol prevented these memory deficits, as well as non-spatial working memory disruptions that were assessed by the novel object recognition test. This is based on the innate preference of rats to examine novel objects rather than familiar ones [72,84] and is primarily used to evaluate the effects of drugs on memory [85], not involving primary reinforcements or stressful cues, such as food deprivation and/or electric shocks [86-88].

CONCLUSIONS

Our results provide novel evidence for the protective ability of resveratrol when administered before injury in the hypoxic-ischemic neonatal brain by ameliorating the morphological damage, especially in the cortex and in the hippocampus, and preventing rats from experiencing long-lasting functional damage. We postulate that one of the mechanisms by means of which resveratrol protects against hypoxic-ischemic damage may be the protection of mitochondrial inner membrane integrity and the maintenance of transmembrane potential, as well as the reduction of ROS production. A more profound understanding of the molecular mechanisms of action of this polyphenol in the hypoxic-ischemic model will require further investigation.

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11.PREDOCTORAL RESEARCH STAY / EGONALDI PREDOKTORALA

EFFECTS OF ERYTHROPOIETIN ON SUPEROXIDE DISMUTASE TRANSGENIC MICE AFTER NEONATAL HYPOXIC-ISCHEMIC BRAIN INJURY

INTRODUCTION AND AIMS

The main goal of the present work was to determine if the delayed administration of erythropoietin (Epo) in superoxide dismutase (SOD) overexpressors can overcome effects of high H_2O_2.

Perinatal hypoxic-ischemic injury causes devastating brain damage associated with significant morbidity and mortality. There is a little doubt about the fact that oxidative stress is one of the mayor contributor to hypoxic-ischemic brain injury, since oxygen radicals are overproduced and the defense mechanisms are altered. The cell´s most important defense mechanism against the excessive production of three radicals is the concerted action of three enzymes: superoxide dismutase, glutathione peroxidase and catalase. SOD catalyzes the dismutation of the superoxide radical to hydrogen peroxide. Catalase and glutathion peroxidase catalyze the reduction of H_2O_2 to water and oxygen.

This group has previously described that in this neonatal model of hypoxic-ischemic brain injury, mice transgenic (tg) for SOD 1 had greater brain damage than their non- transgenic (ntg) littermates. The neonatal brain in the SOD 1 tg mouse accumulates H_2O_2 in response to HI, contributing to cell death, as H_2O_2 is known to be toxic to neurons as well as other cell type. H_2O_2 convert to the highly toxic hydroxyl radical in the presence of iron via the Fenton reaction. The immature brain has relatively high levels of iron in response to HI. The endogenous GPx and catalase are low in neonates, which may explain the increased accumulation of H_2O_2 in SOD overexpressors in the settings of HI and subsequently the greater damage of injury seen in these animals. So GPx and catalase are incapable of ameliorating the deleterious effects of an overproduction of H_2O_2.

We have reproduced the hypoxic-ischemic brain injury following the Rice-Vannucci method in P9 mice, consisting in the permanent ligation of the left common carotid artery, and after a period of recovery (90 minutes), we asphyxiated them for 45 minutes in an environment of 10% O_2. As the combination of ischemia and hypoxia produces an area of infarct ipsilateral to the ligation, we compared both side of the brain, especially at the level of median hippocampus.

Once we induced the HI injury, we administered the Epo, three doses of 5 U/g, immediately after HI, at 24 hours and after 5 days. Epo is a glycoprotein that has been shown to reduce the infarct volume and improve short-term sensorimotor outcomes. So we had six groups:

We determined whether 3 doses of epo well ameliorate the injury or enhancing the repair by measuring volumes by MRI and volumetrically, performing Nissl and Perl´s Iron staining, as both together increases the ability of visualize dead and dying cells, and carrying out some immunostaining with epo and epo-receptor and hif 1alpha and hif 2 alpha, to study the HIF-EPO pathway.

ESPECIFIC AIMS

- To determine whether exogenous Epo will protect the brain from severe injury by overcoming the downregulation of HIF from high H_2O_2.
- Delayed administration of Epo in SOD overexpressors to determine whether exogenous Epo can overcome effects of high H_2O_2.

MATERIAL AND METHODS

ANIMALS AND ETHICS STATEMENTS

All animal research was approved by the *Institutional Animal Care and Use Committee* at the University of California San Francisco and performed with the highest standards of humane care as set forth in the "Guide for the Care and Use of Laboratory Animals", U.S. Dept. of Health and Human services, 85-23, 1985.

Mice were anesthetized with pentobarbital (100 mg/kg) and perfused through the left ventricle with cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), five days after the HI procedure. The brains were removed and post-fixed in the same fixative for four hours, then transferred to 30% sucrose in 0.1 M phosphate buffer. Coronal sections were cut through the forebrain at 50 μm intervals on a Vibratome (Ted Pella, Inc). Alternate sections were stained with cresyl violet and with Perl's iron stain, mounted onto gelatinized slides, dehydrated and coverslipped with Permount.

CRESYL VIOLET

Cresyl violet is a standard histological stain for neurons. Sections are first mounted onto gelatin coated slides and dried over night. Neonatal brains do not need to be delipidized, and after a rinse in H_2O, slides are immersed in stain for 3-5 min. Three components are made and then mixed together: 0.3 g cresyl echt violet (Roboz) in 50 mL H_20, 3.48 mL glacial acetic acid in 300 mL H_2O and 5.44 g sodium acetate in 200 mL H_20. Slides are then rinsed 2x in H_20, differentiated in 70% ethanol with a few drops of acetic acid, followed by dehydration in graded ethanols, 2 changes of xylene, and coverslipped with Depex (Biomedical Specialties, Santa Monica, CA).

PERL'S IRON STAIN

Perl's iron stain enhanced with DAB.[13] Free floating sections are incubated for 30 min in 2% potassium ferrocyanide (Sigma) mixed 1:1 with 2% hydrochloric acid, rinsed 3x in H_2O, then reacted with diaminobenzidine (DAB) (20 mg/10mL PB, 13.3 ul 30% H_2O_2). When DAB is visibly deposited in injured areas of tissue, the reaction is

stopped by rinsing sections 3x in H_20. Sections are then mounted onto gelatin coated slides, allowed to dry, dehydrated in graded ethanols and coverslipped.

QUANTIFICATION OF DAMAGE

Brains were scored, in a masked fashion, for degree of injury using both cresyl violet and Perl's stains. All sections were examined, and eight regions of the brain were scored: the anterior, middle and posterior cortex, CA1, CA2, CA3 and dentate gyrus of the hippocampus, and caudate putamen, with the contralateral side serving as a reference for uninjured tissue. Each region was given a score of 0-3 such that 0 = no detectable neuronal loss, 1 = small focal areas of neuronal loss, 2 = columnar damage in the cortex involving predominately layers II-IV and/or moderate cell loss in the hippocampus, and 3 = cystic infarction and gliosis. The score for each region was then added for a final score ranging from 0 – 24.

RESULTS

EXPERIMENTAL GROUPS (n=20) 3 litters

1. WT animals HI (n=1)
2. WT animals HI + Vehicle (n=4)
3. WT animals HI + Epo (n=5)

4. SOD+ animals HI (n=2)
5. SOD+ animals HI + Vehicle (n=4)
6. SOD+ animals HI + Epo (n=5)

GENOTYPING

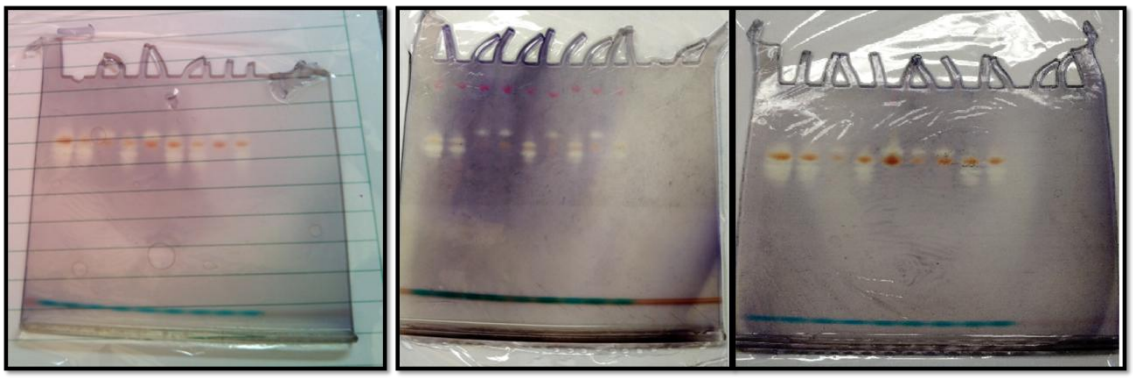


Figure 1: Genotyping.

DEATH RATIO

1st litter: 0
2nd litter: 1 (SOD, eaten by mother)
3rd litter: 2 (SOD during ischemia, and SOD+EPO eaten by mother)

In the first and the second litters they were with just the mum.
In the third litter both mum and dad were present.

4 animals of 20 → 20%

BODY AND BRAIN WEIGHT

There are not statistically significant differences between the groups, the n is not big enough.

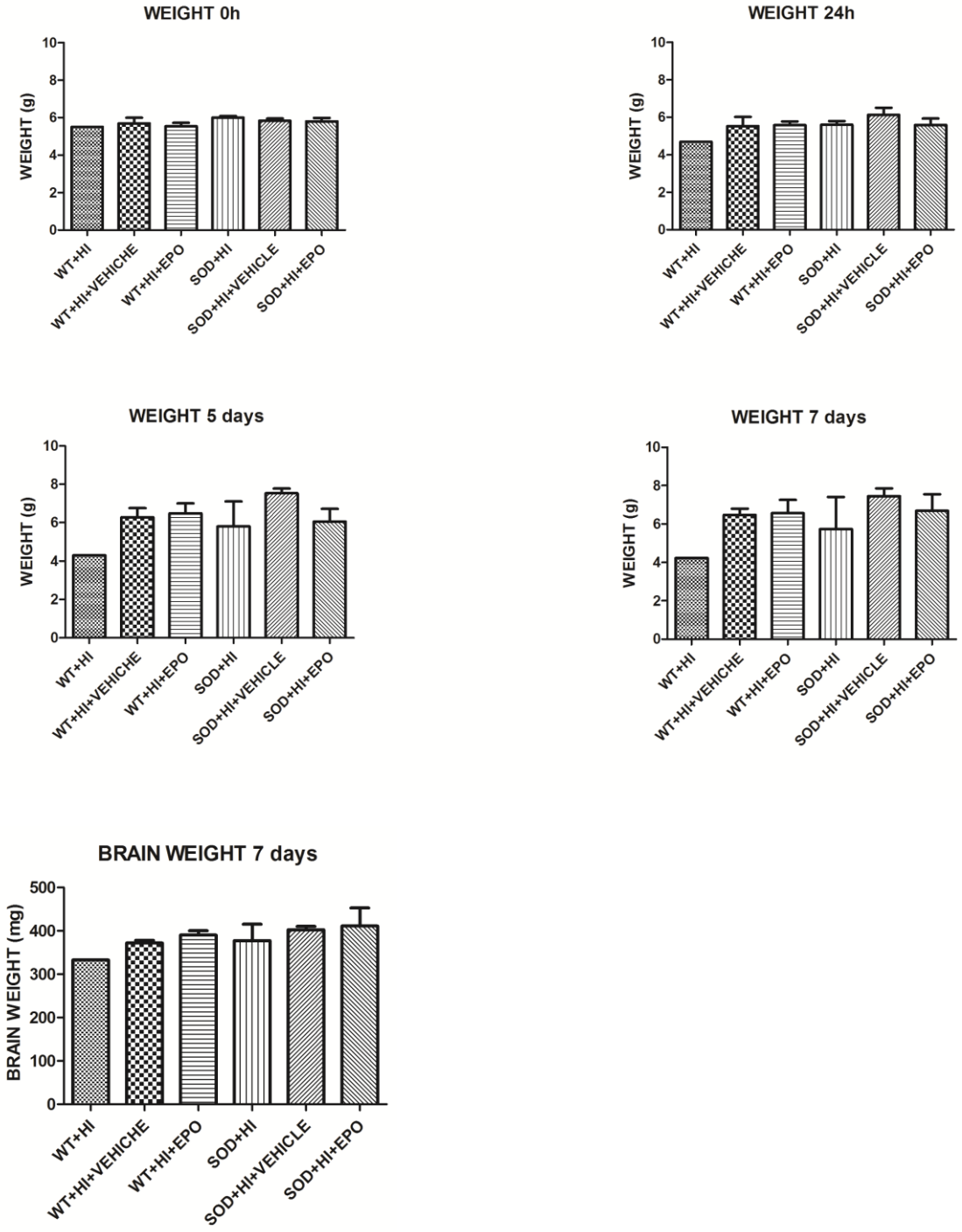


Figure 2: Body weight (g) on P9, P10, P14 and P16 and brain weight on P16 animals (mg). There are not statistically significant differences among the groups, the n is not big enough.

Table 1: Animals, treatment, sex, hypoxia-ischemia date, body weight at different time points and brain weight.

ANIMAL, TREATMENT and SEX	HYPOXIA-ISCHEMIA (P9)	WEIGHT 0 h	WEIGHT 24 h	WEIGHT 5 days	WEIGHT 7 days (perfusion)	BRAIN WEIGHT 7 days
HI 4 SOD+ VEH (M)	07/31/15	5.9 g	6.3 g	8 g	7.91 g	415.4 mg
HI 5 WT+EPO (M)	07/31/15	5.5 g	5.6 g	7 g	7.28 g	370.9 mg
HI 2 SOD+VEH (M)	07/31/15	5.6 g	5.1 g	6 g	6.43 g	390.1 mg
HI 6 SOD+EPO (F)	07/31/15	6 g	6.6 g	7.8 g	8.13 g	423.4 mg
HI 9 SOD+EPO (M)	07/31/15	5.9 g	6.4 g	7.5 g	7.91 g	565.9 mg
HI 3 WT+VEH (M)	07/31/15	5.7 g	5.1 g	5.5 g	5.72 g	356.7 mg
HI 1 SOD (F)	07/31/15	5.9 g	5.8 g	7.1 g	7.4 g	415.2 mg
HI 8 WT	07/31/15	Burnt				
HI 7 SOD (F)	07/31/15	For Breeding				
HI 8 WT +EPO (F)	08/03/15	5.0 g	5.4 g	7.1 g	7.61 g	404.8 mg
HI 1 SOD+VEH (M)	08/03/15	5.8 g	6.2 g	7.9 g	8.23 g	417.2 mg
HI 6 WT+EPO (F)	08/03/15	5.3 g	5.5 g	7.1 g	7.4 g	394.6 mg
HI 4 WT+VEH (F)	08/03/15	5.5 g	5.1 g	5.8 g	6.25 g	367.9 mg
HI 5 SOD (F)	08/03/15	5.8 g	Eaten by mother			
HI 2 SOD+EPO (F)	08/03/15	6.1 g	5.8 g	7.3 g	8.02g	373.3
HI 3 WT+VEH (M)	08/03/15	5.1 g	4.9 g	6.1 g	6.64 g	383.3 mg
HI 7 SOD+EPO (M)	08/03/15	4.9 g	4.5 g	5. 0 g	5.62 g	356.8 mg
HI 7 WT+VEH (M)	08/05/15	6.5 g	7.0 g	7.6 g	7.26 g	382.9 mg
HI 1 SOD (M)	08/05/15	6.1 g	5.4 g	4.5 g	4.05 g	339.4 mg
HI 9 SOD+EPO (M)	08/05/15	6.2 g	5.4 g	4.5 g	Dead	
HI 3 WT (M)	08/05/15	5.5 g	4.7 g	4.3 g	4.23 g	332.8 mg
HI 8 SOD+EPO (M)	08/05/15	5.8 g	4.9 g	4.2 g	3.77	333.8 mg
HI 4 SOD+VEH (F)	08/05/15	6.1 g	6.9 g	7.2 g	7. 25	386.4 mg
HI 5 WT+EPO (F)	08/05/15	6.0 g	6.3 g	6.8 g	6.65 g	389.5 mg
HI 6 WT+EPO (F)	08/05/15	5.9 g	5.1 g	4.4 g	3.89 g	351.5 mg
HI 2 SOD (F)	08/05/15	5.8 g	Dead			

QUANTIFICATION OF DAMAGE

ANN´S DAMAGE SCORE

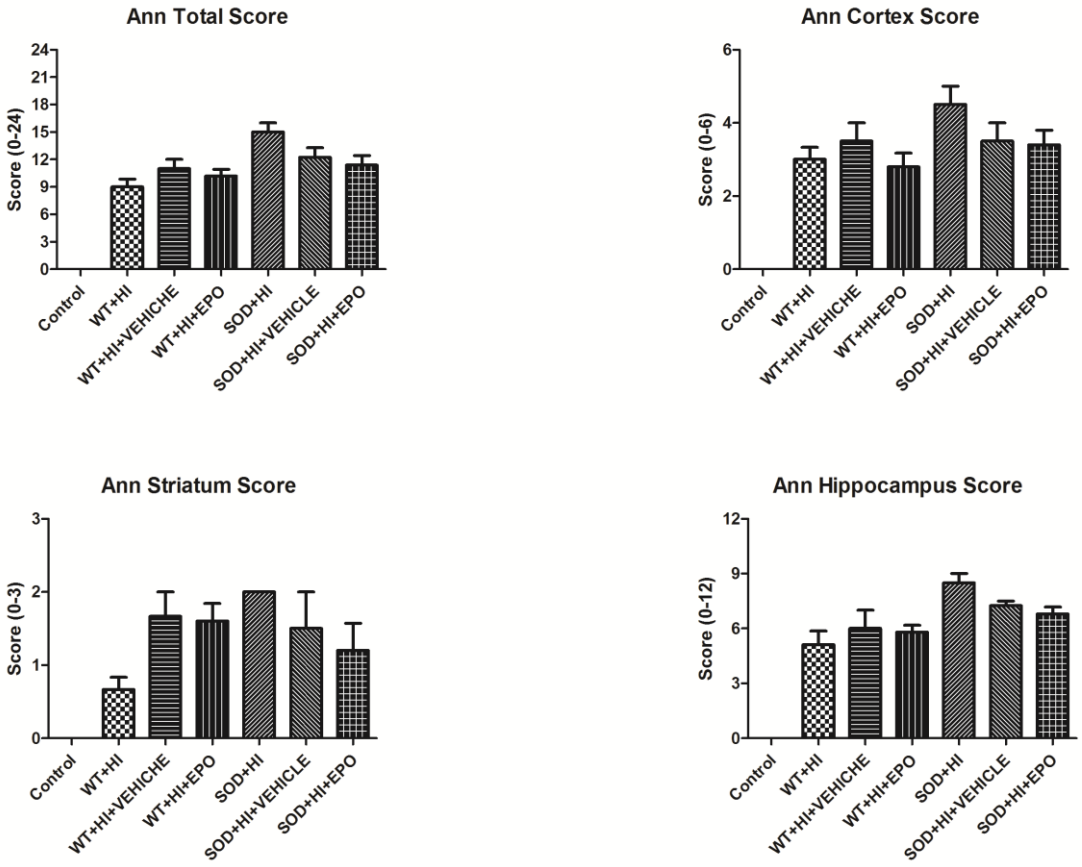


Figure 3: Damage Score measured by Ann. Total damage score, in the hippocampus, in the cortex and in the striatum. There are not statistically significant differences among the groups, the n is not big enough.

MY DAMAGE SCORE

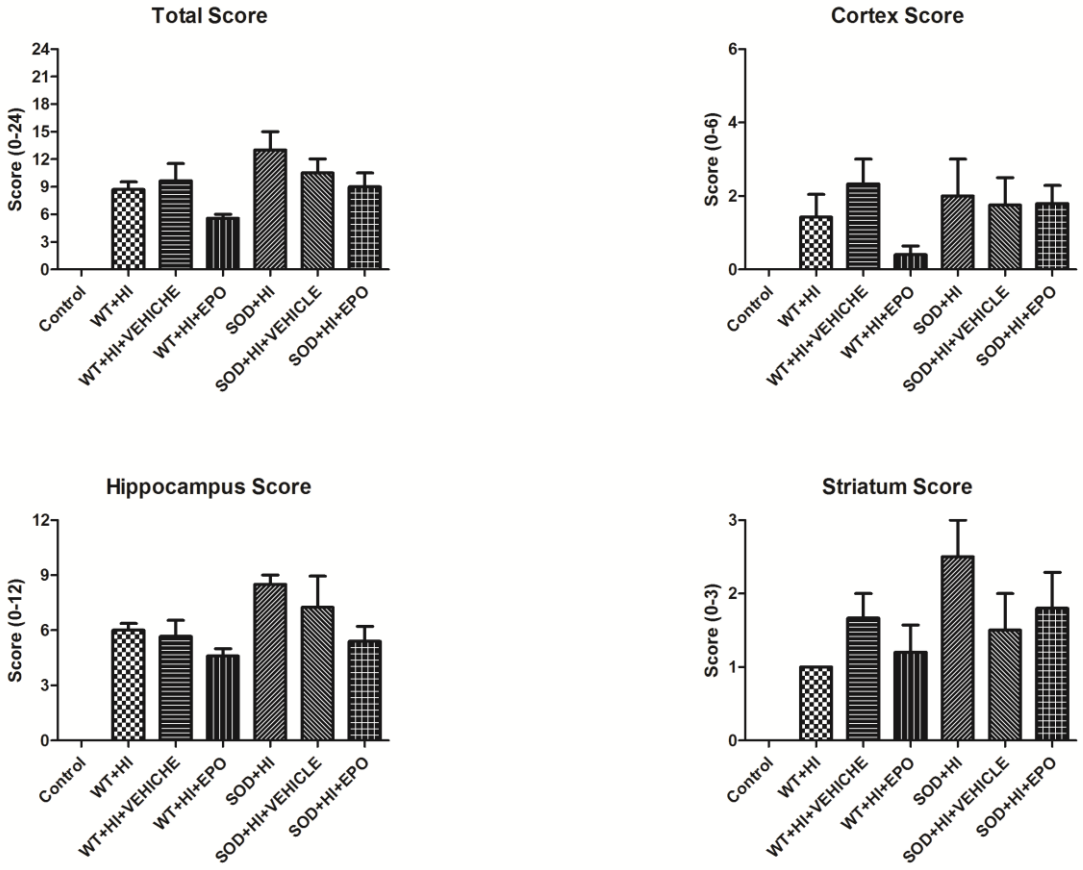


Figure 4: Damage Score measured by Olatz. Total damage score, in the hippocampus, in the cortex and in the striatum. There are not statistically significant differences between the groups, the n is not big enough.

HISTOLOGICAL EVALUATION

- **WT GROUPS**
  - **CRESYL VIOLET STAINING**

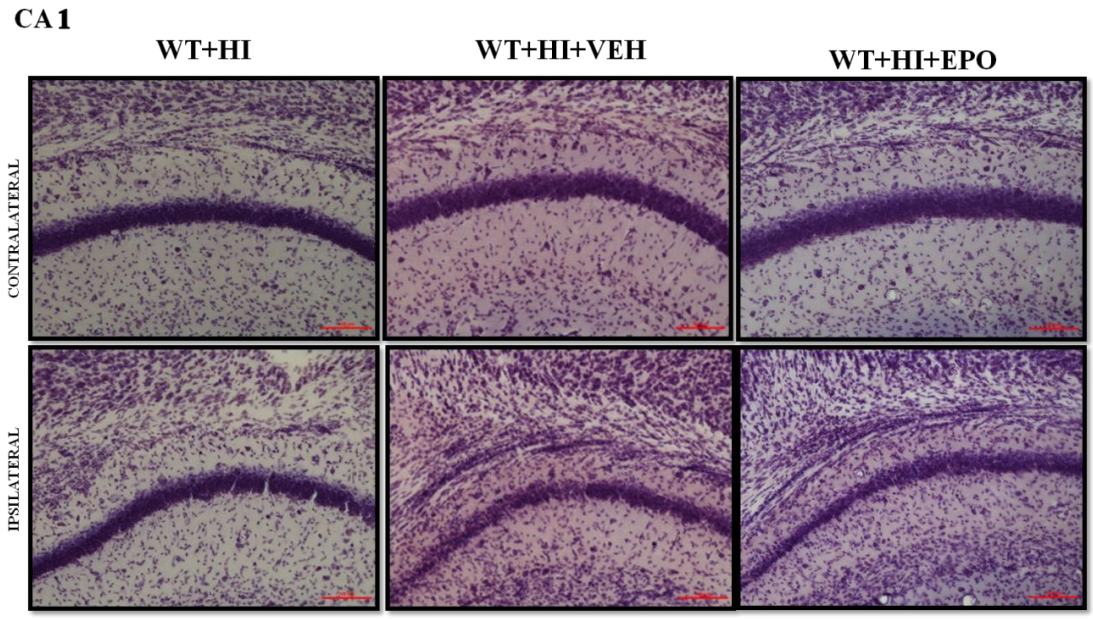


Figure 5: Representative microphotographs of Nissl-stained brain sections of different experimental groups (wild type) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of CA 1 area of the hippocampus. Scale bar: 100 µm.

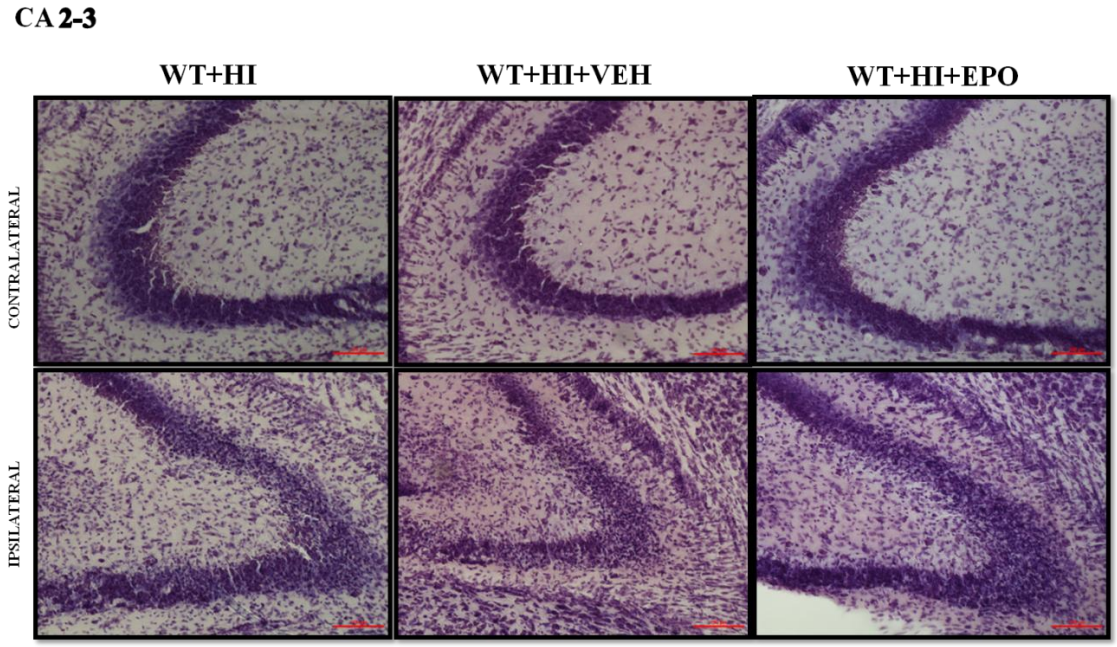


Figure 6: Representative microphotographs of Nissl-stained brain sections of different experimental groups (wild type) seven days after hypoxia-ischemia (P15)

obtained from contralateral and ipsilateral sides of CA 2-3 area of the hippocampus. Scale bar: 100 µm.

DG

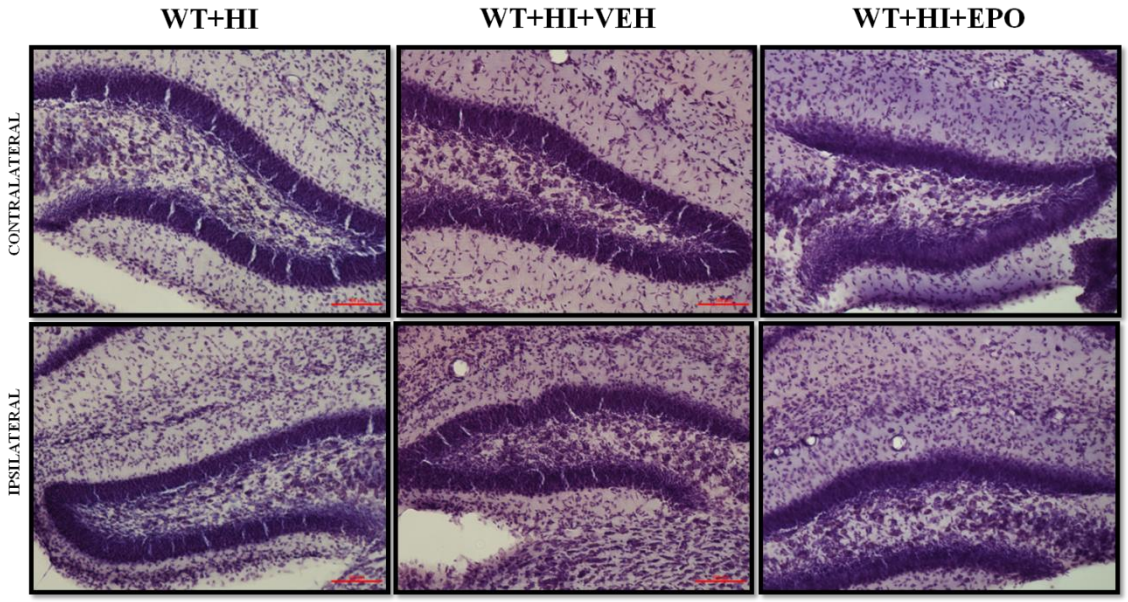


Figure 7: Representative microphotographs of Nissl-stained brain sections of different experimental groups (wild type) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of dentate gyrus area of the hippocampus. Scale bar: 100 µm.

CTX

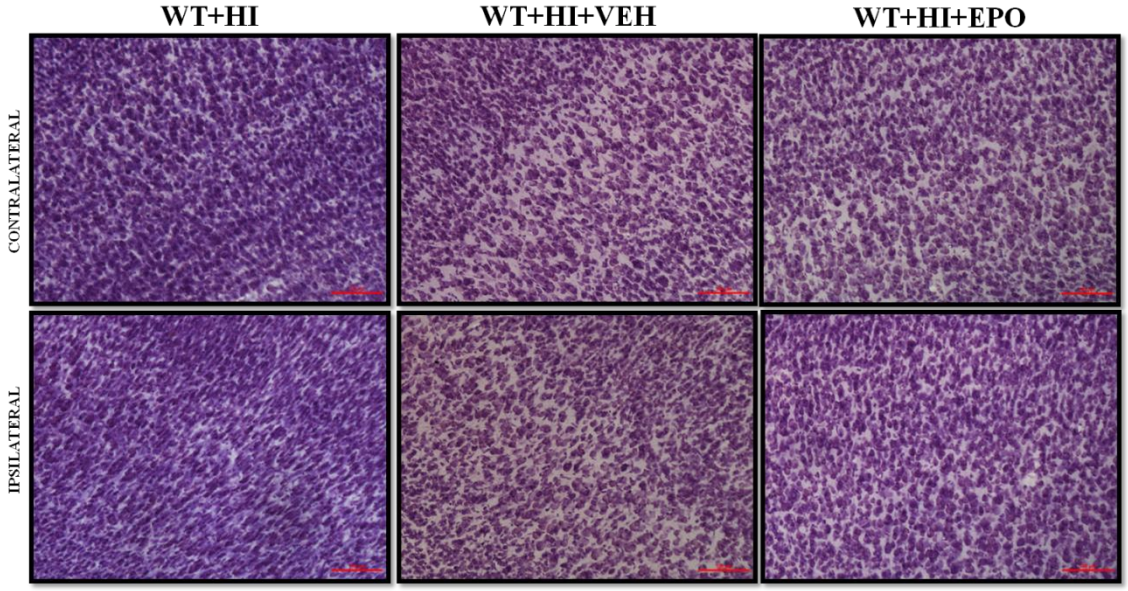


Figure 8: Representative microphotographs of Nissl-stained brain sections of different experimental groups (wild type) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of parietal cortex. Scale bar: 100 µm.

- **PERL´S IRON STAINING**

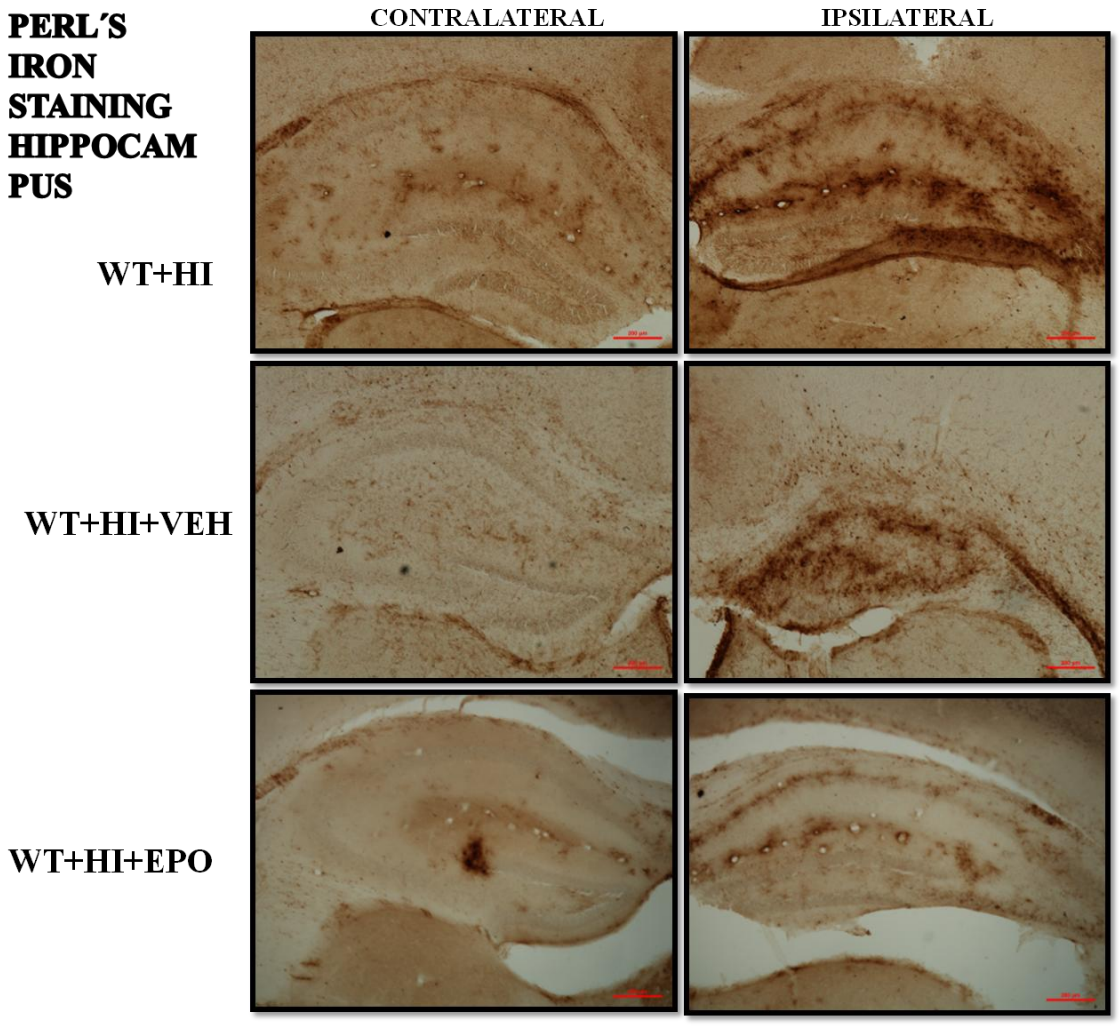


Figure 9: Representative microphotographs of Perl´s Iron stained brain sections of different experimental groups (wild type) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the hippocampus. Scale bar: 200 µm.

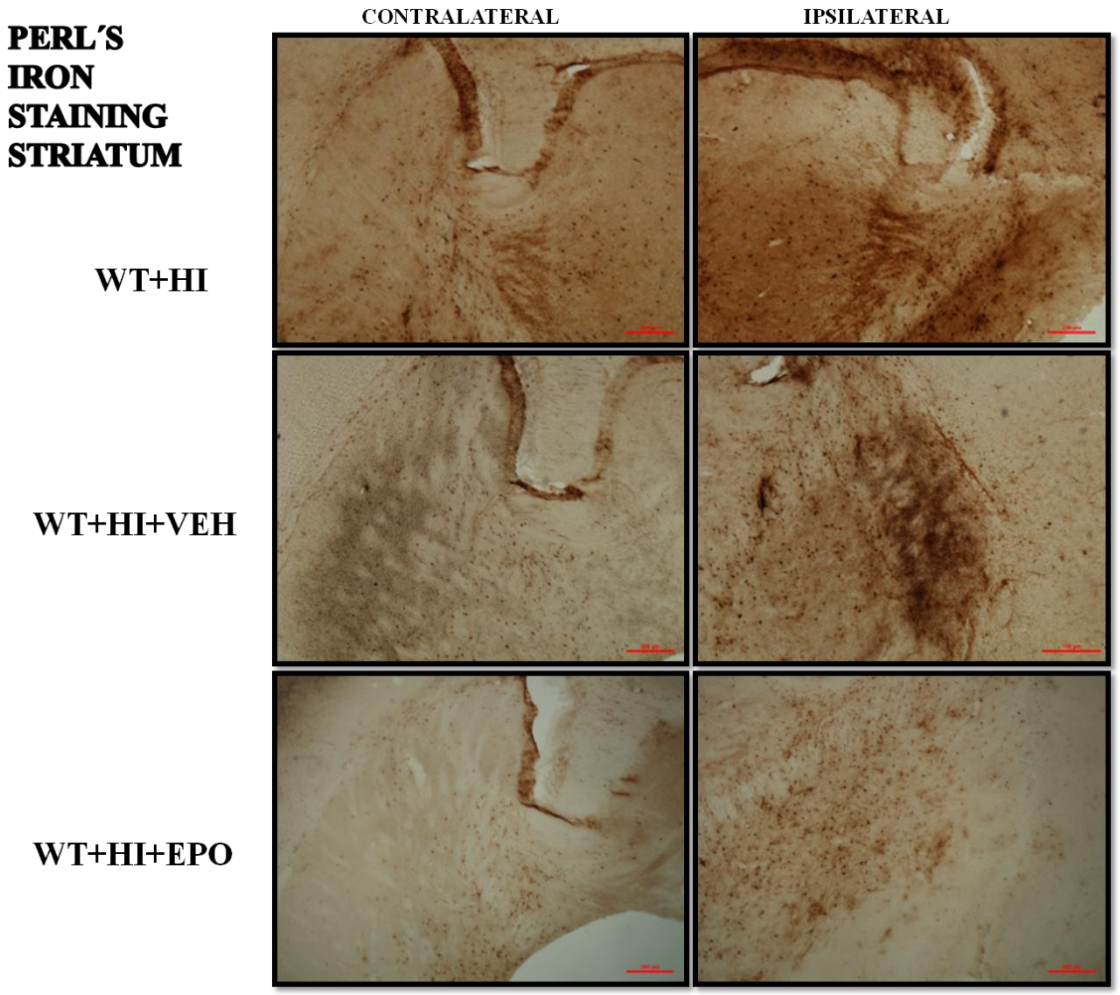


Figure 10: Representative microphotographs of Perl´s Iron stained brain sections of different experimental groups (wild type) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the striatum. Scale bar: 200 µm.

- **SOD groups**

  - **CRESYL VIOLET STAINING**

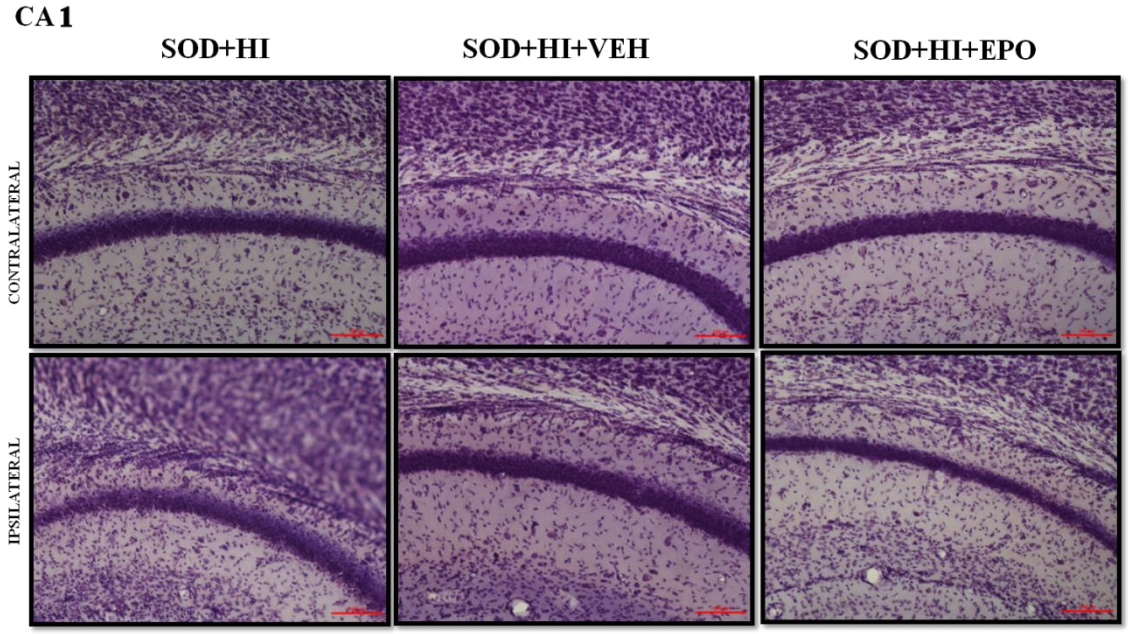


Figure 11: Representative microphotographs of Nissl-stained brain sections of different experimental groups (SOD+) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of CA 1 of the hippocampus. Scale bar: 100 µm.

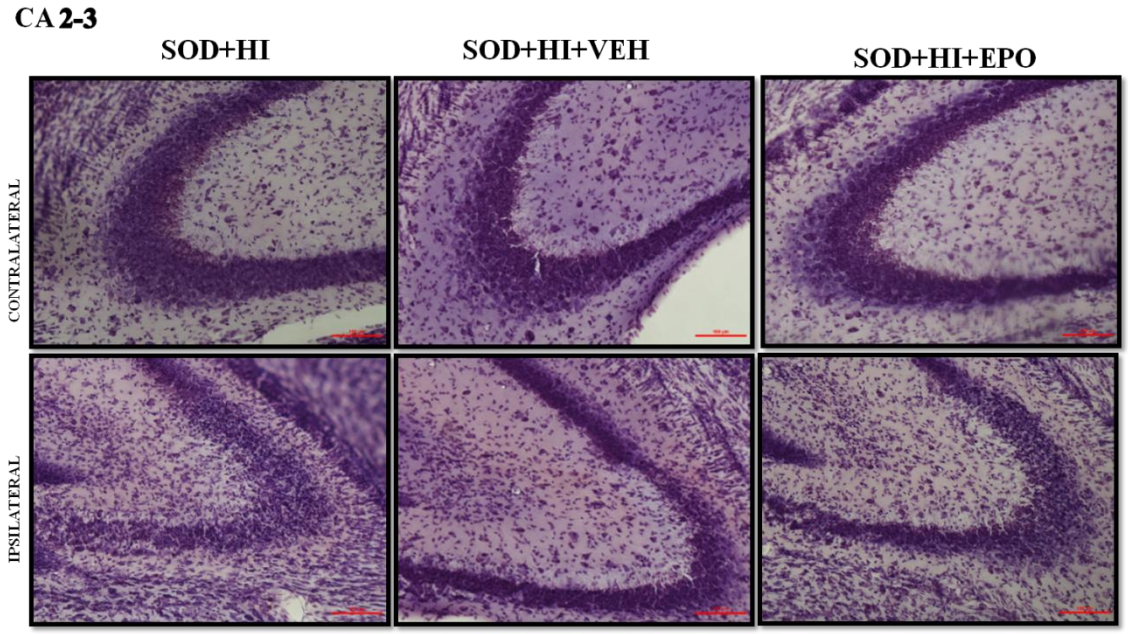


Figure 12: Representative microphotographs of Nissl-stained brain sections of different experimental groups (SOD+) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of CA 2-3 of the hippocampus. Scale bar: 100 µm.

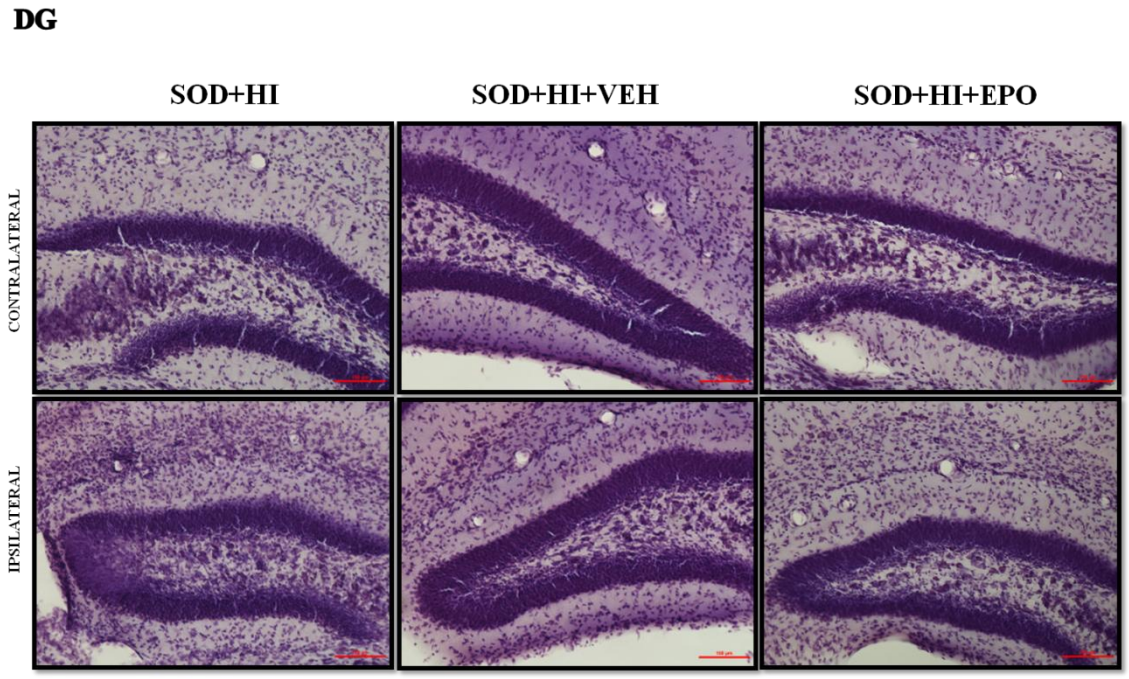


Figure 13: Representative microphotographs of Nissl-stained brain sections of different experimental groups (SOD+) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of dentate gyrus of the hippocampus. Scale bar: 100 µm.

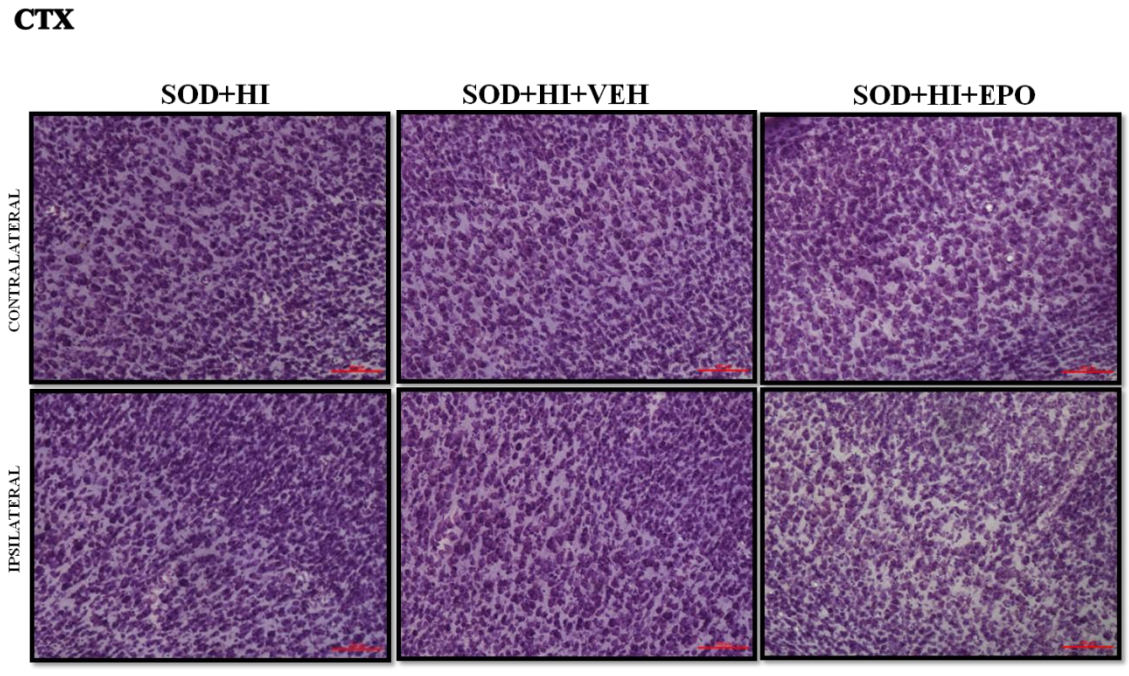


Figure 14: Representative microphotographs of Nissl-stained brain sections of different experimental groups (SOD+) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the parietal cortex. Scale bar: 100 µm.

- **PERL´S IRON STAINING**

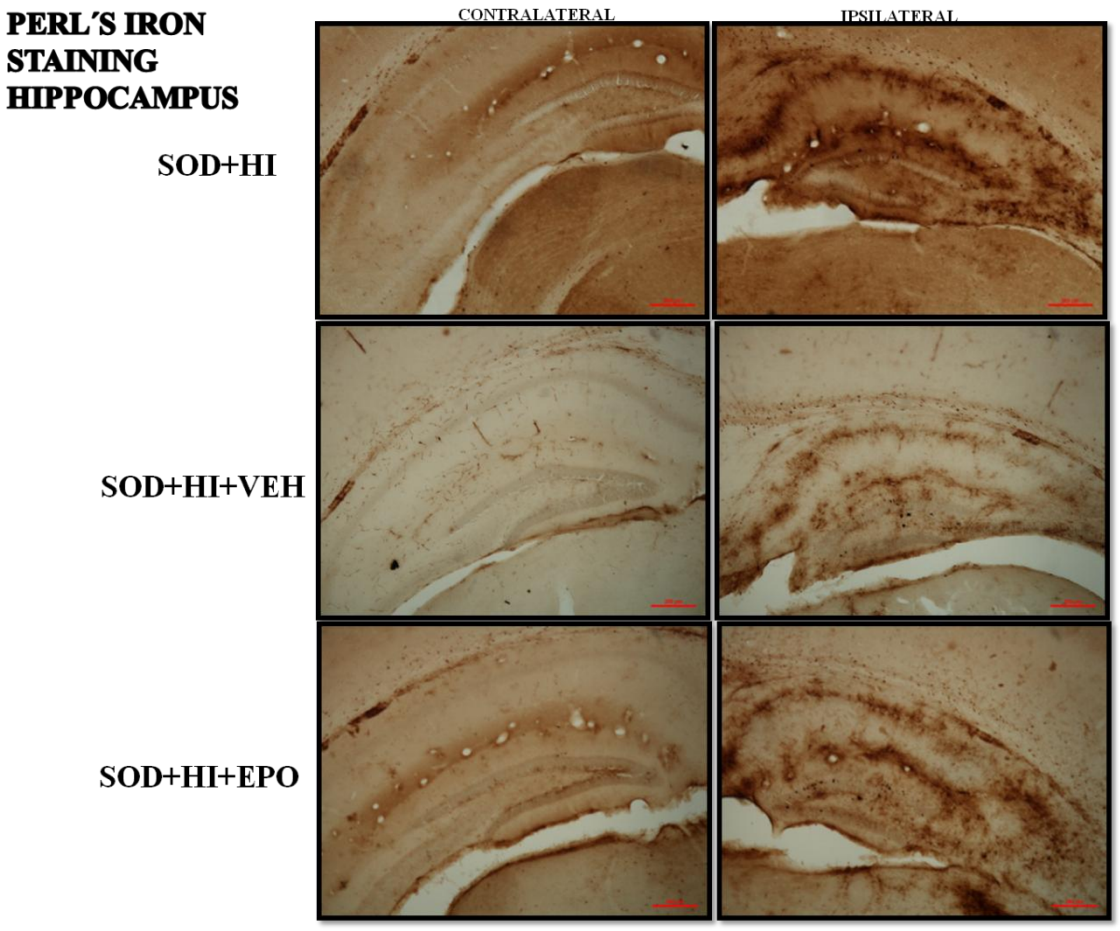


Figure 15: Representative microphotographs of Perl´s Iron stained brain sections of different experimental groups (SOD+) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the hippocampus. Scale bar: 200 µm.

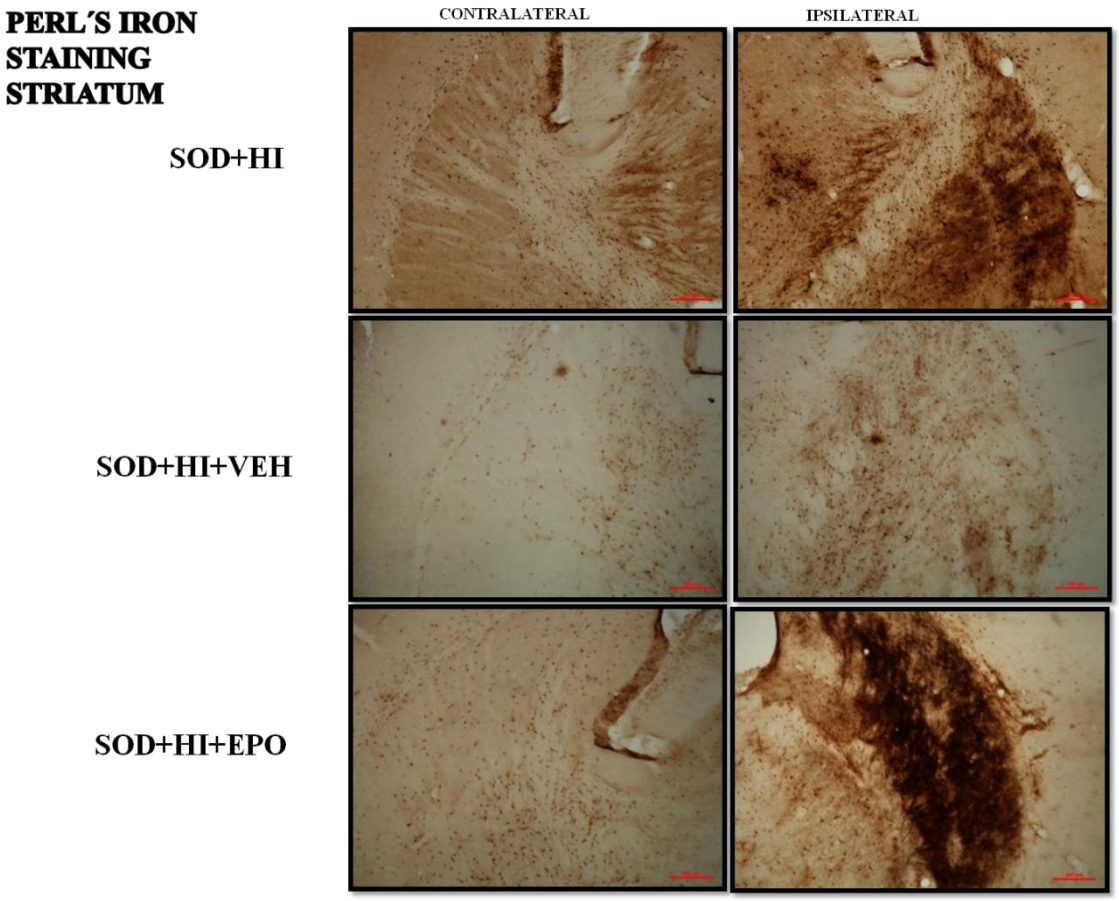


Figure 16: Representative microphotographs of Perl´s Iron stained brain sections of different experimental groups (SOD+) seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the striatum. Scale bar: 200 µm.

- **ALL TOGETHER**
  - **CRESYL VIOLET STAINING**

CA 1

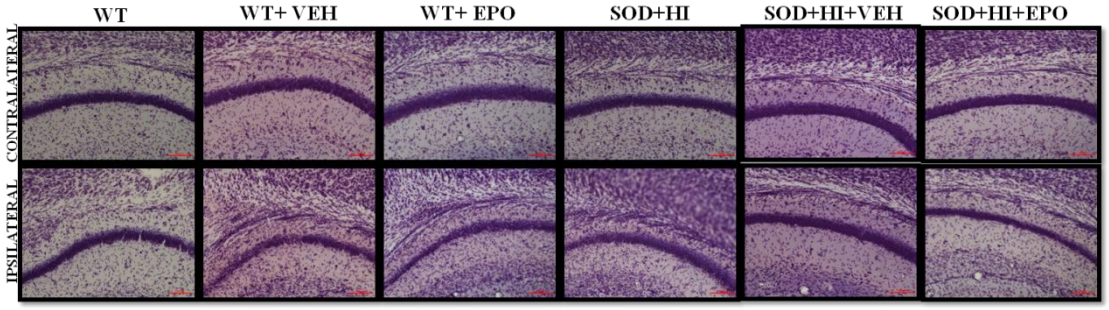


Figure 17: Representative microphotographs of Nissl-stained brain sections of all the experimental groups seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of CA 1 of the hippocampus. Scale bar: 100 µm.

CA 2-3

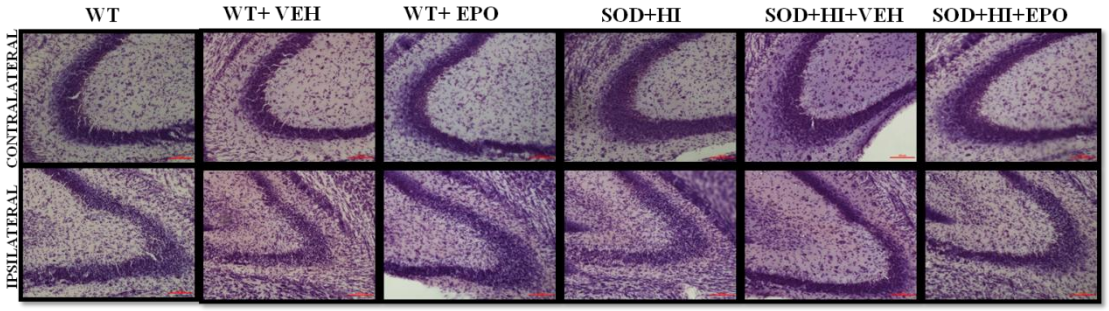


Figure 18: Representative microphotographs of Nissl-stained brain sections of all the experimental groups seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of CA 2-3 of the hippocampus. Scale bar: 100 µm.

DG

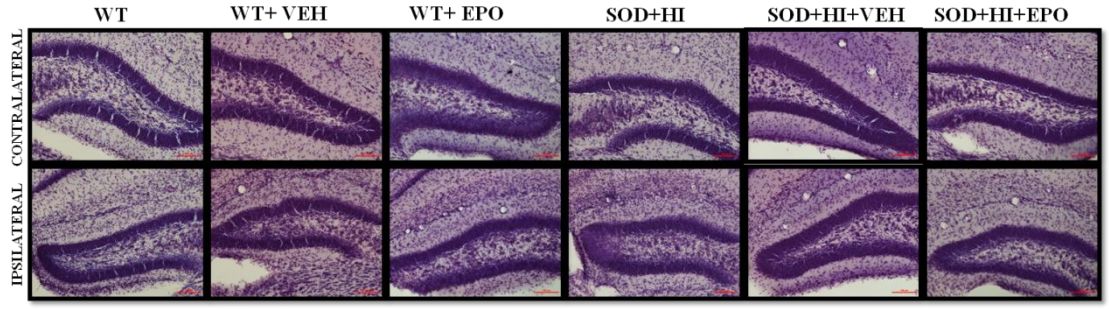


Figure 19: Representative microphotographs of Nissl-stained brain sections of all the experimental groups seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of dentate gyrus of the hippocampus. Scale bar: 100 µm.

CTX

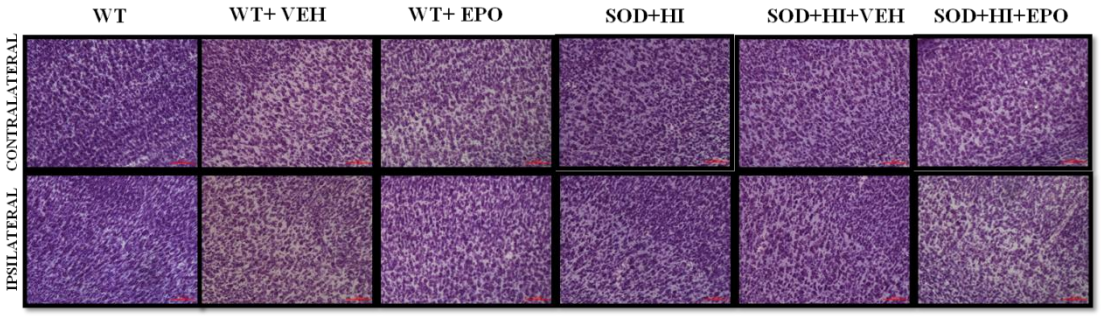


Figure 20: Representative microphotographs of Nissl-stained brain sections of all the experimental groups seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the parietal cortex. Scale bar: 100 µm.

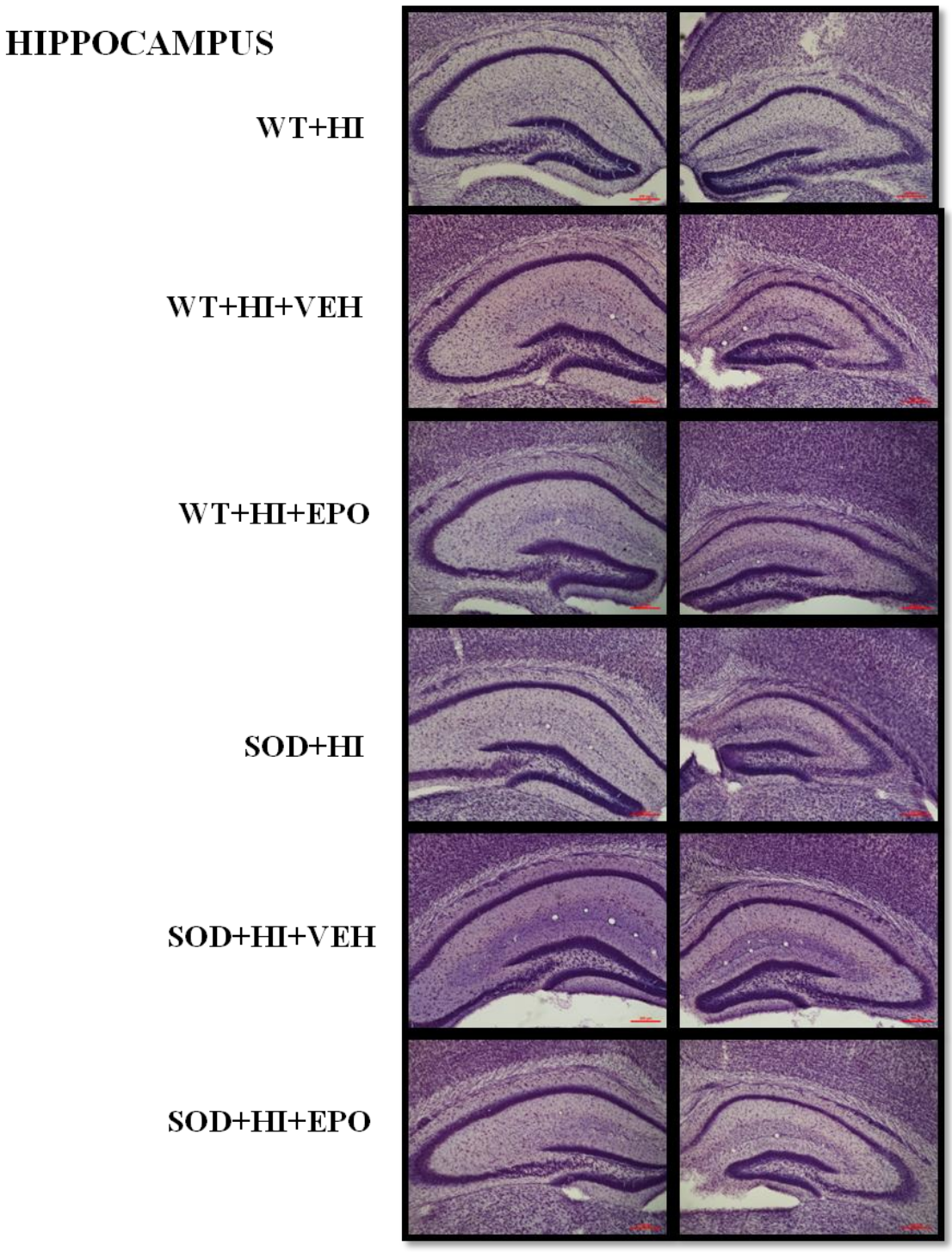


Figure 21: Representative microphotographs of Nissl-stained brain sections of all the experimental groups seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the hippocampus. Scale bar: 200 µm.

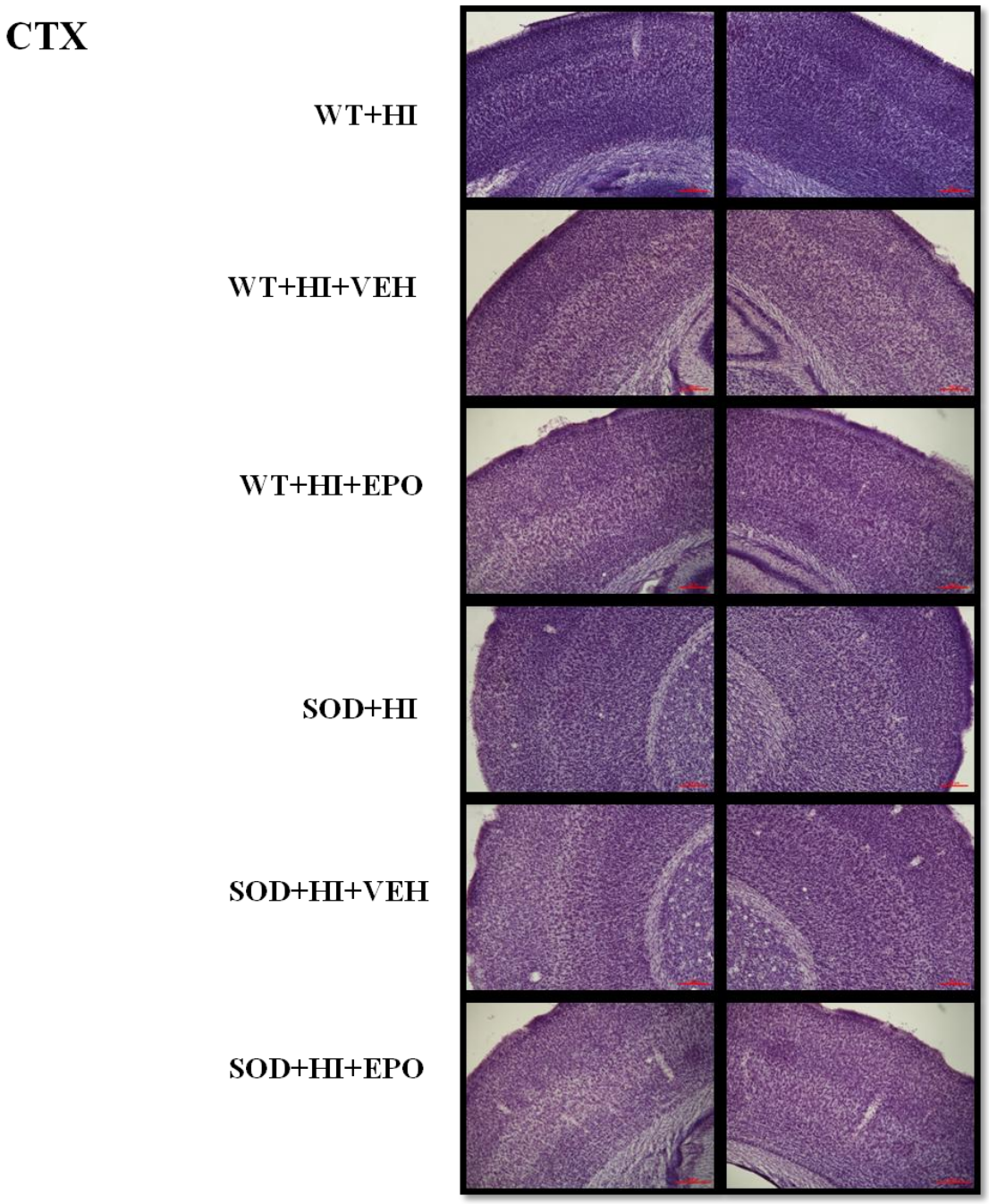


Figure 22: Representative microphotographs of Nissl-stained brain sections of all the experimental groups seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the parietal cortex. Scale bar: 200 µm.

- **PERL´S IRON STAINING**

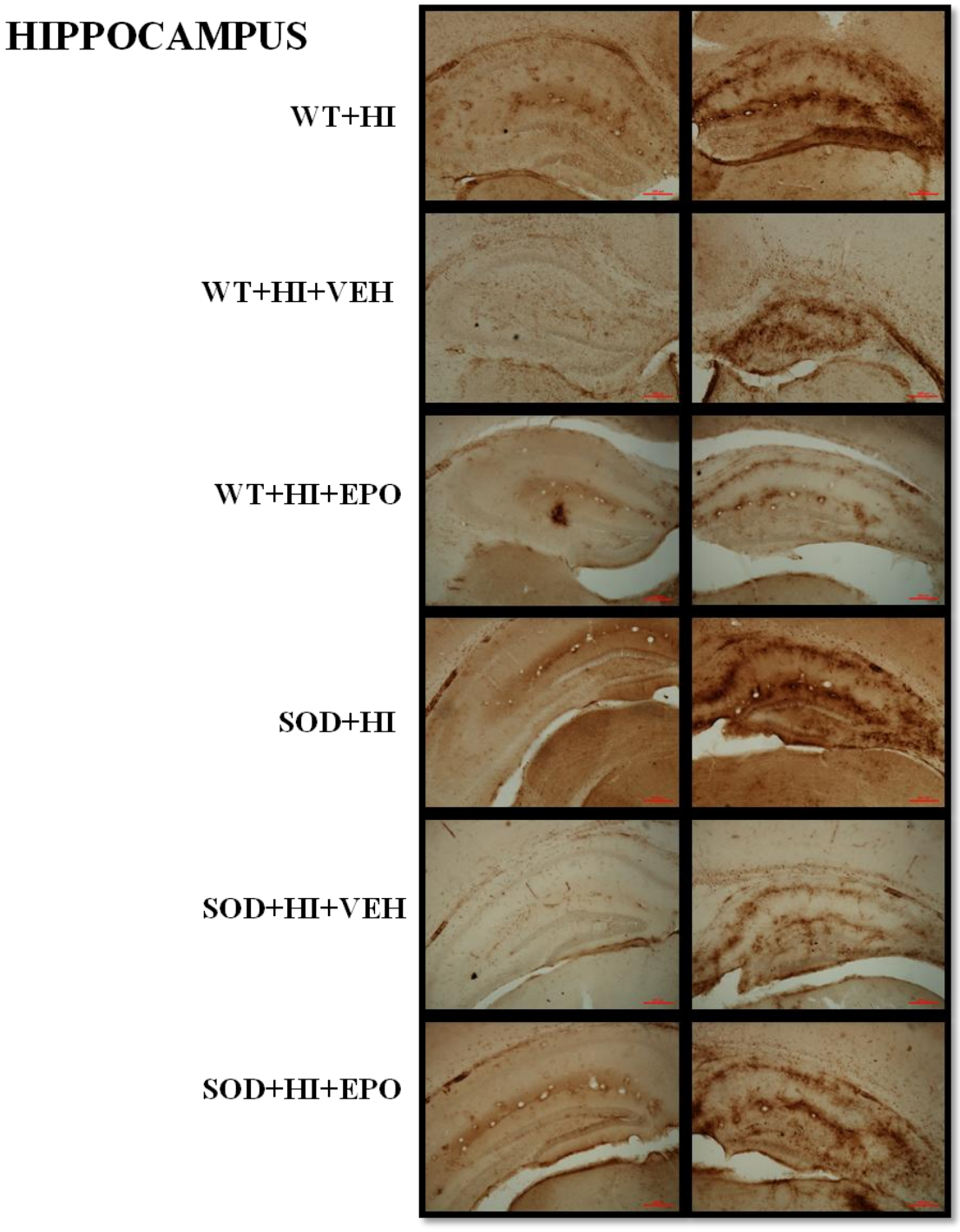


Figure 23: Representative microphotographs of Perl´s Iron-stained brain sections of all the experimental groups seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the hippocampus. Scale bar: 200 µm.

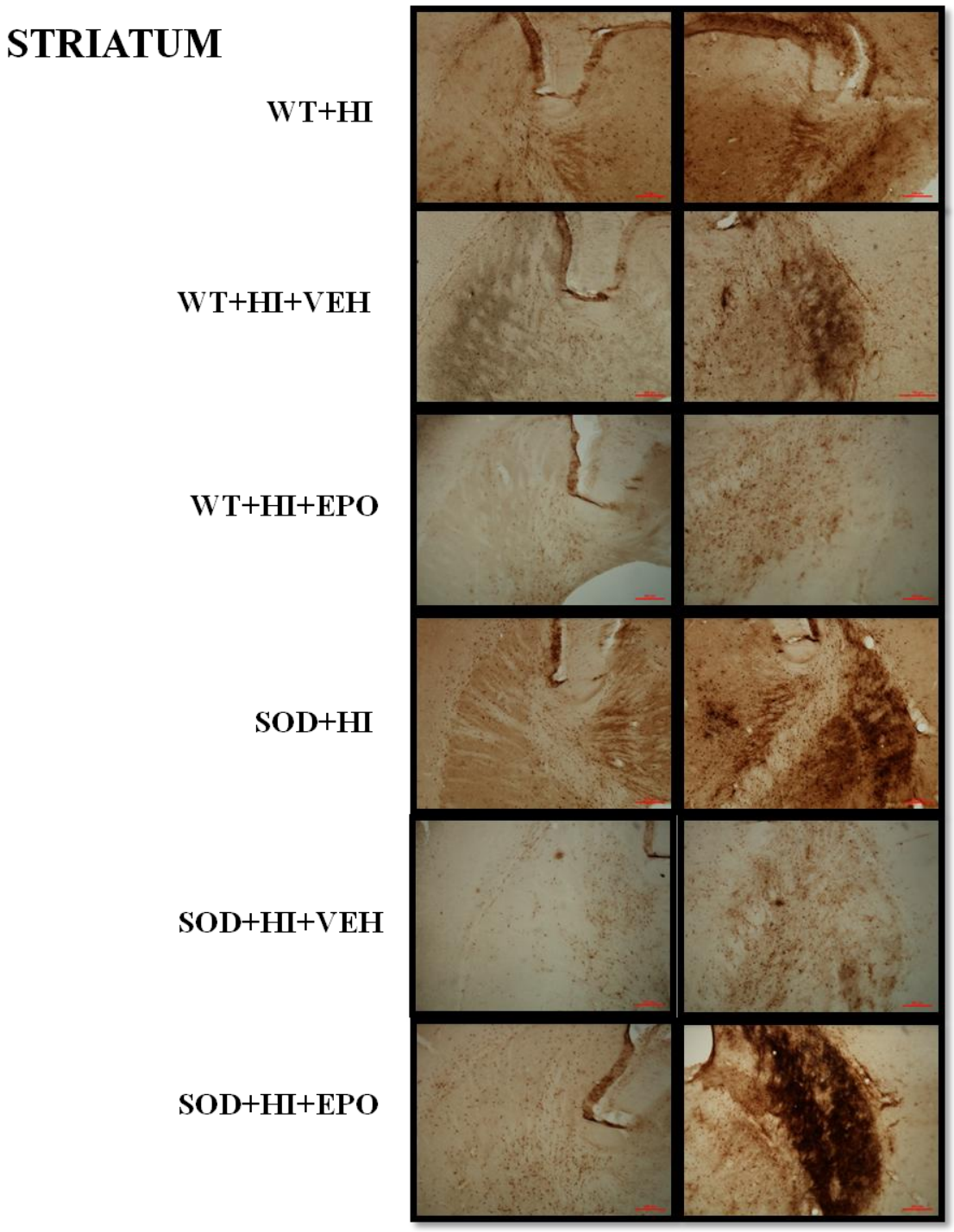


Figure 24: Representative microphotographs of Perl´s Iron-stained brain sections of all the experimental groups seven days after hypoxia-ischemia (P15) obtained from contralateral and ipsilateral sides of the striatum. Scale bar: 200 µm.

ERITROPOIETINAREN EFEKTUAK SUPEROXIDO DISMUTASARAKO TRANSGENIKOAK DIREN SAGUETAN BURMUIN-KALTE HIPOXIKO-ISKEMIKOAREN OSTEAN

SARRERA ETA HELBURUAK

Proiektu honen helburua eritropoietinaren (Epo) administrazio berantiarrak superoxido dismutasa (SOD) entzima gain-espresatzen duten saguetan hipoxia-iskemiare ostean sortutako hidrogeno peroxidoa (H_2O_2) kontzentrazio altuak gainditu ahal dituen aztertzea da.

Kalte hipoxiko-iskemiko perinatalak burmuineko lesio konponezinak eragin ditzake, umeengan heriotzaren eta epe luzeko desgaitasunen kausa nagusiena delarik. Estres oxidatzailea hipoxia-iskemiaren osteko burmuin-kaltearen eragile nagusitzat hartzen da, oxigeno erradikal ugari ekoizten direlako eta aldi berean, babeserako mekanismoek behar bezala funtzionatzen ez dutelako. Gehiegizko erradikal askeen ekoizpenaren kontra egiteko zelularen babes-sistema nagusiena hiru entzima antioxidatzaileen aldi bereko elkarrekintzek osatzen dute, superoxido dismutasa, glutation peroxidasa eta katalasa entzimena, hain zuzen ere. Superoxido dismutasak erradikal superoxidoaren dismutasioa katalizatzen du, hidrogeno peroxidoa sortuz. Katalasak eta glutation peroxidasak, berriz, H_2O_2-ren erreduzioa katalizatzen dute, ura eta oxigenoa sortuz.

Donna M. Ferrieroren taldeak aurretik deskribatu izan du hipoxia-iskemia neonatalaren animalia eredu batean SOD 1 generako transgenikoak diren saguek bere kumaldi bereko kide ez-transgenikoek (wild type) baino garuneko kalte handiagoa aurkezten dutela. SOD 1 generako transgenikoa den burmuin neonatalak hipoxia-iskemiaren aurrean H_2O_2 metatzen du, zelulen heriotza sustatuz, izan ere H_2O_2 toxikoa da bai neuronentzat zein beste zelula-motentzat ere. H_2O_2 erradikal hidroxilo bilakatzen da burdinaren presentzian Fenton erreakzioaren bitartez. Garatu gabeko burmuinak hipoxia-iskemiaren ondorioz burdin kantitate handiak ditu. Era berean, jaioberrietan glutation peroxidasa eta katalasa entzima antioxidatzaileen mailak urriak dira, honek azalduko luke zergatik H_2O_2 metatzen den SOD gain-espresatzen duten saguen burmuinetan eta ondorioz zergatik duten garuneko kalte larriagoa ez transgenikoek baino. Hortaz, glutation peroxidasa eta katalasa ez dira gai H_2O_2 gehiegizko ekoizpenaren efektuak murrizteko.

MATERIALAK ETA METODOAK

ANIMALIAK ETA ETIKA IKERKETAN

Ikerketa honetan erabilitako animaliak saguaen (*Mus musculus*) C57BL/6J anduia izan ziren. Arratoiei eginiko prozedura esperimental guztiak San Frantzisko Kaliforniako Unibertsitateko (UCSF) ikerketako eta animalien maneiurako argibideak jarraituz egin ziren. Era berean, prozedura eta protokolo esperimental guztiek unibertsitate honetako Animalien Ongizaterako Etika Batzordearen onarpena jaso zuten.

Animaliak unibertsitate honetako animaliategian mantendu ziren, 12 orduko argi eta iluntasun zikloekin, ura eta janaria edozein momentutan hartzeko askatasunarekin, eta oinazeak, sufrimenduak, larritasunak edo lesio luzeak saihesteko eta animalia kopurua ahalik eta gehien murrizteko neurriak hartu izan zirelarik

PROZEDURA EXPERIMENTALAK: KALTE HIPOXIKO-ISKEMIKOA

9 egun zituztenean (P9) saguei hipoxia-iskemia (HI) eragin zitzaien Rice-Vannucci eredua jarraiki. Burmuin-kalte hipoxiko-iskemikoa eragiteko, lehenengo eta behin, ezkerrezko arteria karotida komuna behin-betiko okluitu egin zitzaien eta ordu eta erdiko atseden prozesu baten ostean, saguak 45 minutuz asfixitu genituen oxigeno kontzentrazioa %10ra jaitsiz. Iskemia eta hipoxiaren konbinazioak burmuinean infartua ekartzen du okluitu dugun aldearekiko ipsilaterala dena, bereziki tarteko hipokanpoaren mailara.

Behin kalte hipoxiko-iskemikoa eragin diegularik, eritropoietinazko 5 U/gramoko hiru dosi sartu genizkien, hipoxia-iskemia bukatu eta jarraian, 24 orduren buruan eta bost egunen ostean. Epo glikoproteina bata da infartuaren bolumena murriztu eta epe laburreko kalte sentsomotorrak hobetzeko gaitasuna duena. Epo PBS 10x esterilean disolbatu genuen, behikulu moduan erabili genuen. Gure talde experimentalak sei izan ziren, animalia guztiei hipoxia-iskemia eragin eginen, eta horietako batzuk behikulua jaso zuten eta gainontzekoek epo:

TALDE EXPERIMENTALAK

1. Animalia ez-transgenikoak, HI (WT+HI)
2. Animalia ez-transgenikoak, HI + behikulua (WT+HI+VEHICLE)
3. Animalia ez-transgenikoak, HI + Epo (WT+HI+EPO)

4. SODrako animalia transgenikoak, HI (SOD+HI)
5. SODrako animalia transgenikoak, HI + behikulua (SOD+HI+VEHICLE)
6. SODrako animalia transgenikoak, HI + Epo (SOD+HI+EPO)

Eritropoietinazko hiru dosiak lesioa leuntzeko eta konpontzeko gaitasuna duten aztertu genuen, Nissl eta Perlen burdinazko tindaketak egin genituen, biak batera erabiliz zelula hilak eta kaltetuak ikusteko erraztasuna ematen dutelako, eta kuantifikazioa egin genuen.

Ikerketa histologiko animaliak injekzio intraperitoneal baten bidez hil ziren hipoxia-iskemia eragin eta 7 egunen ostean (P16), pentobarbial sodiko eutanasikoa 20g/100ml kontzentrazioan sartuz. Erabili beharreko bolumena animaliaren pisuaren arabera kalkulatu zen, 40 ml animalia 100 gramoko. Animaliak intrakardiakoki perfunditu egin ziren, bonba peristaltiko baten bitartez, zeinak likido garbitzailea presioa eta fluxua konstantean pasarazten zuen. Behin animalia lokartzen genuela, bihotzeko ezkerreko aurikula mozten genion finkatze-soluzioaren (paraformaldehidoa %4ean) eta odolaren igarotzea ahalbidetuz.Behin paraformaldehidoarekin perfusioa bukatuta, garezurra trepanatu zen artazi eta punta laueko pintzak erabiliz burmuina ateratzeko. Garuna paraformaldehidoan mantendu zen 4 ºC-tan 24 orduz askoz jota. Finkatzeak zelula barneko elementuen insolubilizazioa ahalbidetzen du eta ehunari trinkotasuna ematen dio errazago maneiatu ahal izateko, ehunaren endekapena saihestuz. Finkatzearen ostean, sakarosa %30ean duen buffer fosfatoan (PB) 0,1M jartzen dira ehuna hondoratzen den arte, burmuina kriobabesteko helburuarekin. Lagin hauetatik 50 µm-ko ebakin lodiak eskuratuko ditugu bibratomoa (Ted Pella, Inc) erabiliz, ebakinak PBS 0,1M-tan jasotzen dira tindaketak eta inmunohistokimika egin arte.

NISSL TINDAKETA

Parafinan inkluituriko ebakinak kresil bioletarekin tindatzeko, lehenengo pausua desparafinatzea izan zen horretarako xiloletan sartu ziren 10 minutuko bi bainutan, zelulen mintzak ezegonkortzeko koloratzailearen barneratzea erraztuz. Ondoren, ehuna hidratatu egiten da graduazioan beherantz doazen alkoholetan sartuz berriro hidratatu ziren: minutu bakarreko bainu bana 100º-ko etanoletan, 96º-tan, 70º-tan eta azkenik iturriko uretan. Burmuinen ebakinak tindatzeko 57ºC-tan berotu egin dugun kresil bioletan (kresil bioletazko 0,1 g ur destilatuaren 100 ml-ko, azido azetiko glazialaren 4 tanta gehituta) murgildu genituen. Soberazko koloratzailea kentzeko ur destilatuarekin birritan garbitu ziren laginak. Ehunaren deshidrataziorako gero eta gradu altuagoko etanoletatik (50º, 70º, 96º,100º) pasatu eta azkenik xilenotan sartuko ditugu 10 minutuko bi bainutan. Kontuan hartu behar da 50º, 70º eta 96º-ko alkoholek azido azetiko glaziala % 1ean izan behar dutela ere, desberdintze hobea erdiesteko. Azkenik DPX (Fluka, Sigma-Aldrich) erretxinaz eta porta-estalkiaz estaliko ditugu.

PERLEN BURDIN TINDAKETA

Flotazioan zeuden laginak 30 minutuz potasio ferrozianina %2 (Sigma) eta azido hidroklorikoz egindako nahasketa batean (1:1) inkubatu genituen, ondorioz uretan 3 aldiz garbitzeko. Orduan, diaminobenzidinarekin (DAB) (20 mg/10mL PB, 13.3 ul %30 H_2O_2) erreakzionatu genituen, eta lesioa ikusgai zenean uretan garbiketak burutu genituen. Azkenik, ebakinak porta gelatinizatuetan muntatu, alkoholetan deshidratu eta porta-estalkinekin estali genituen.

KALTEAREN KUANTIFIKAZIO MIKROSKOPIKOA

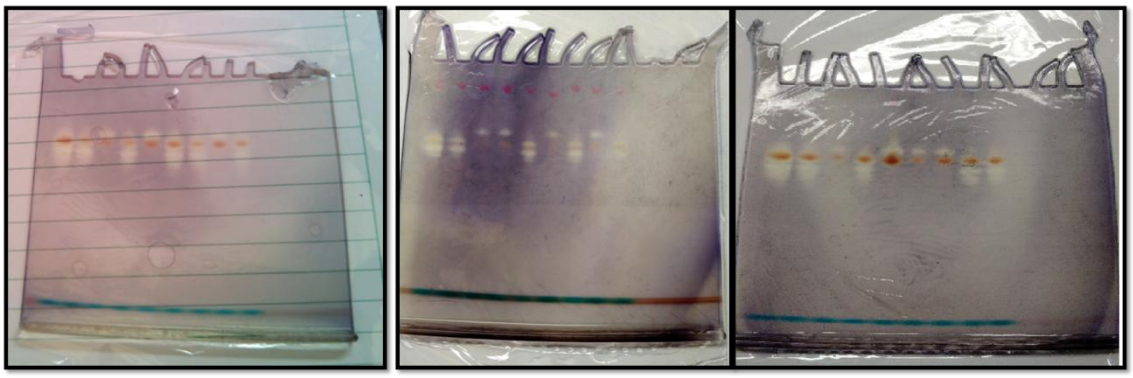
Kuantifikazio mikroskopikorako, Nissl eta Perlen burdin tindaketen osteko laginak mikroskopio optiko batez aztertu eta argazkiak atera genituen, ondoren kalte zelularra aztertu eta kuantifikatzeko sistema histopatologio sasi-kuantitatiboa jarraitu genuelarik (Sheldon eta lank.).

Kortex parietalaren kaltea 0 eta 3 puntu bitartean baloratu zen, aurre, tarteko eta atzeko kortexa azterturik: 0 puntu kalterik ikusi ez zenean, puntu 1 kalteturiko zelula-talde isolatuak, 2 puntu ikusitako kaltea zenbait zelula-taldeek pairatzen zutenean, 3 puntu ehunak infartu moderatu edo larria agertzen zuenean. Estriatumerako balorazio bera jarraitu genuen baina tarteko alderako soilik. Hipokanpoaren kasuan, CA 1, CA 2, CA 3 eta hortz zirkunbalazio zonalde bakoitzeko puntuak gehituta kuantifikatu zen kaltea, beraz, hipokanporako gehinezko kalte-puntuazioa 12 izango litzateke. Ondorioz talde honek garatutako sistema histopatologiko honen bitartez kalteturiko zelulek eduki zezaketen gehienezko puntuazioa 24 puntu ziren.

EMAITZAK

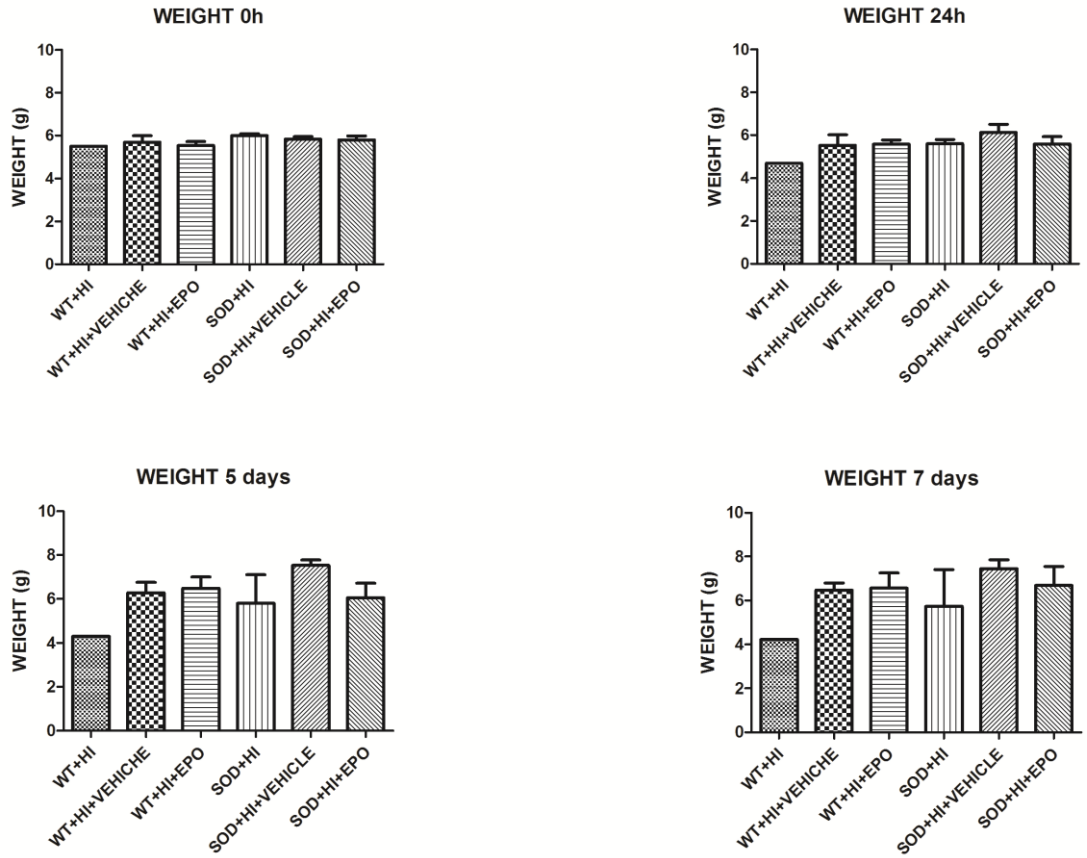
Animalia transgenikoak gutxi izateaz gain, nahiko zaharrak zirenez, arazoak izan nituen talde bakoitzeko animalia nahikoa izateko, ez baitziren gurutzatzen. Hamar bikote gurutzatu bagenituen ere, hiru kumaldi baino ez nituen lortu, horregatik nire n hain txikia da, eta emaitzak ez dira estatistikoki esangarriak. Zailtasun hauek ikusita, beste proiektutan laguntza ematea eskatu zidaten, eta ni izan nintzen ikerlari berriei hipoxia-iskemia zelan eragiten den irakatsi egin diedana. Hala ere, emaitza hauetan ikus dezakegu zelan superoxido dismutasarako transgenikoak ziren animaliak kaltea gehiago azaltzen zutela ez-transgenikoak baino, eta eritropoietina administratzearen ostean burmuinaren kaltea murriztu egiten zela. Hori dela eta, proiektuarekin jarraituko dute, animalia gehiago erosiko dituztenean, izan ere gure hipotesia bete egiten dela iradokitzen dute eskuratutako emaitzek, hots, eritropoietina kaltea leuntzeko gai dela SOD entzima antioxidatzailearen gain-espresioaren ondorioz kaltea handiagoa denean ere.

GENOTIPAZIOA



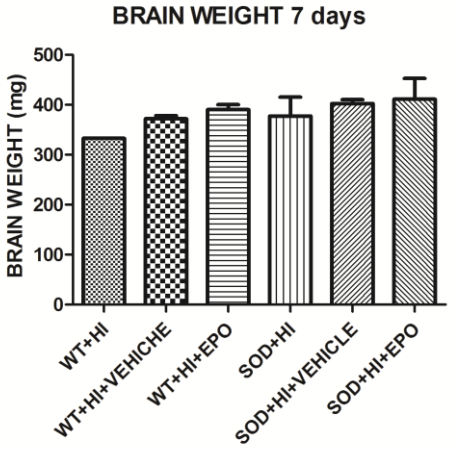
1. irudia: hiru kumaldien genotipazioa.

GORPUTZEKO PISUA



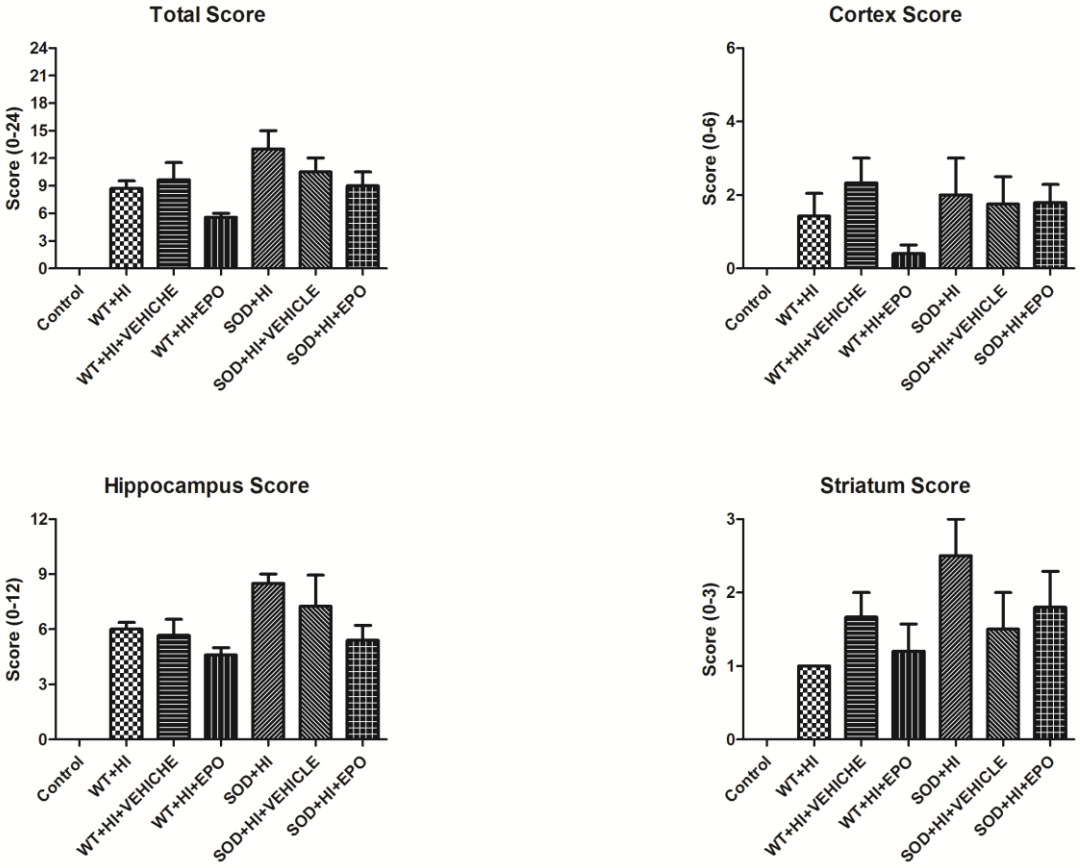
2. Irudia: Talde desberdinetako pisuak gramotan, P9, P10, P14 eta P16 zituztenean, eporen administrazio egunetan eta perfusio-egunean, hain zuzen ere.

BURMUINEKO PISUA



3. irudia: Saguen burmuinen pisua perfusio-egunean, P16an, miligramotan adierazita.

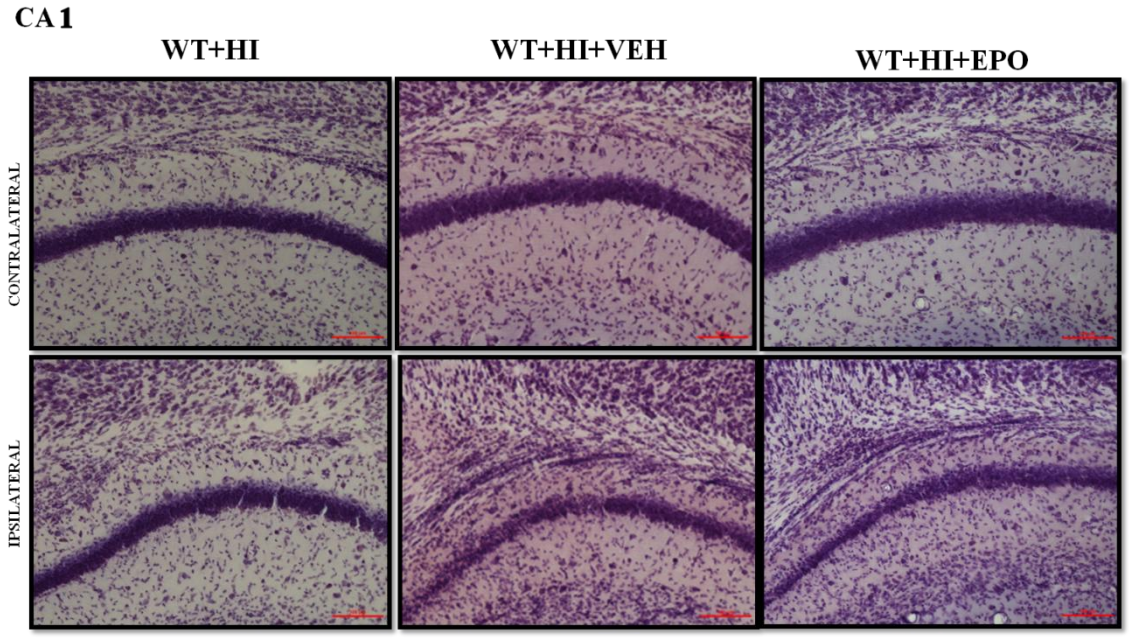
KUANTIFIKAZIOA



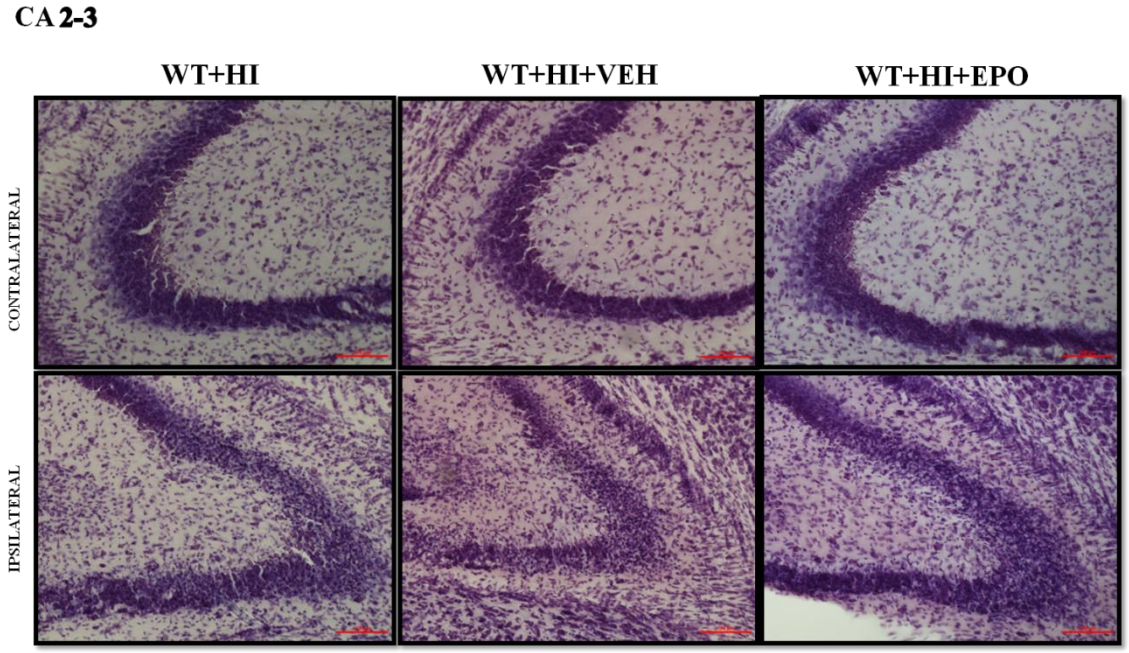
4.irudia: Kaltearen kuantifikazioa, guztira, kortexean, hipokanpoan eta estriatumean animaliek 16 egun dutenean.

EBALUAZIO HISTOLOGIKOA

- **WT TALDEAK (animalia ez-transgenikoetan)**
  - **NISSL TINDAKETA**

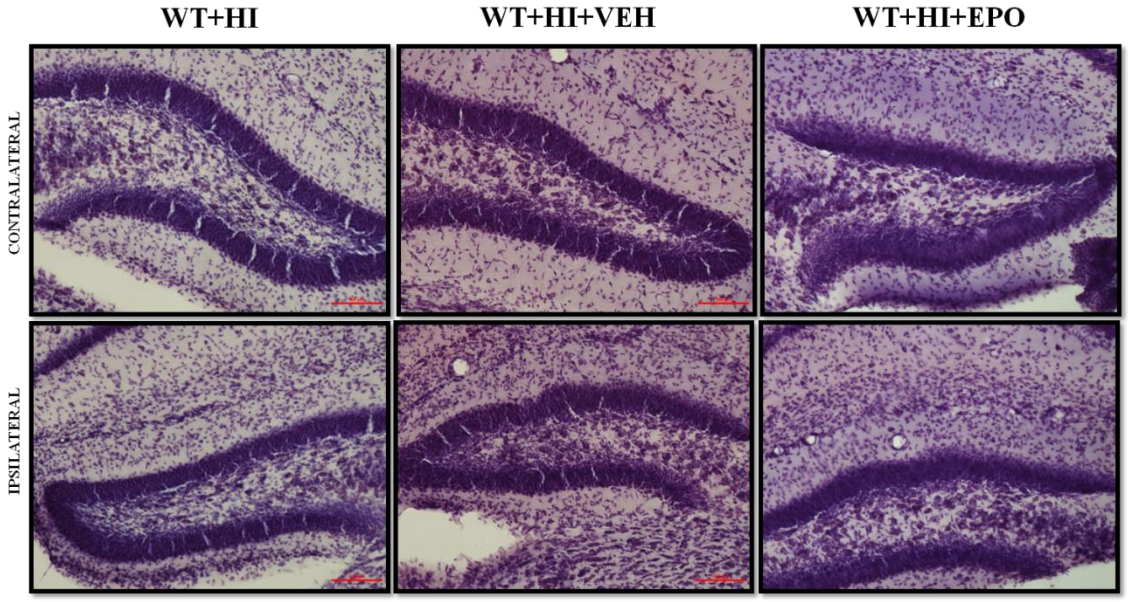


5.irudia: Nisslarekin tindaturiko burmuinaren ebakiak animalia ez-trangenikoetan (wild type), non hipokanpoko CA 1 zonaldeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 100 µm.



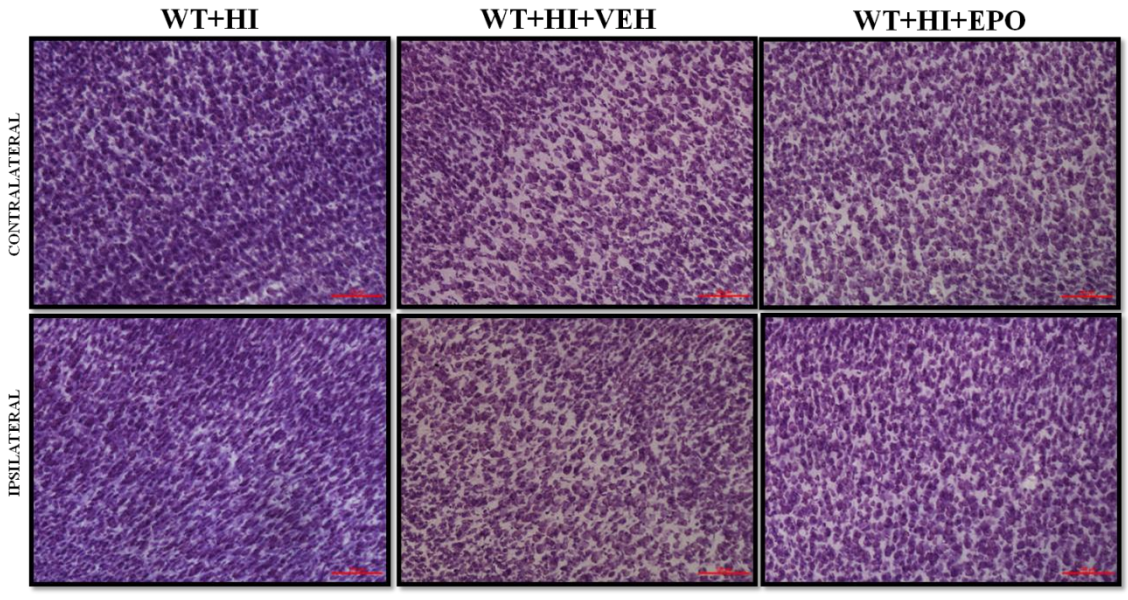
6.irudia: Nisslarekin tindaturiko burmuinaren ebakiak animalia ez-trangenikoetan (wild type), non hipokanpoko CA 2-3 zonaldeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 100 µm.

DG



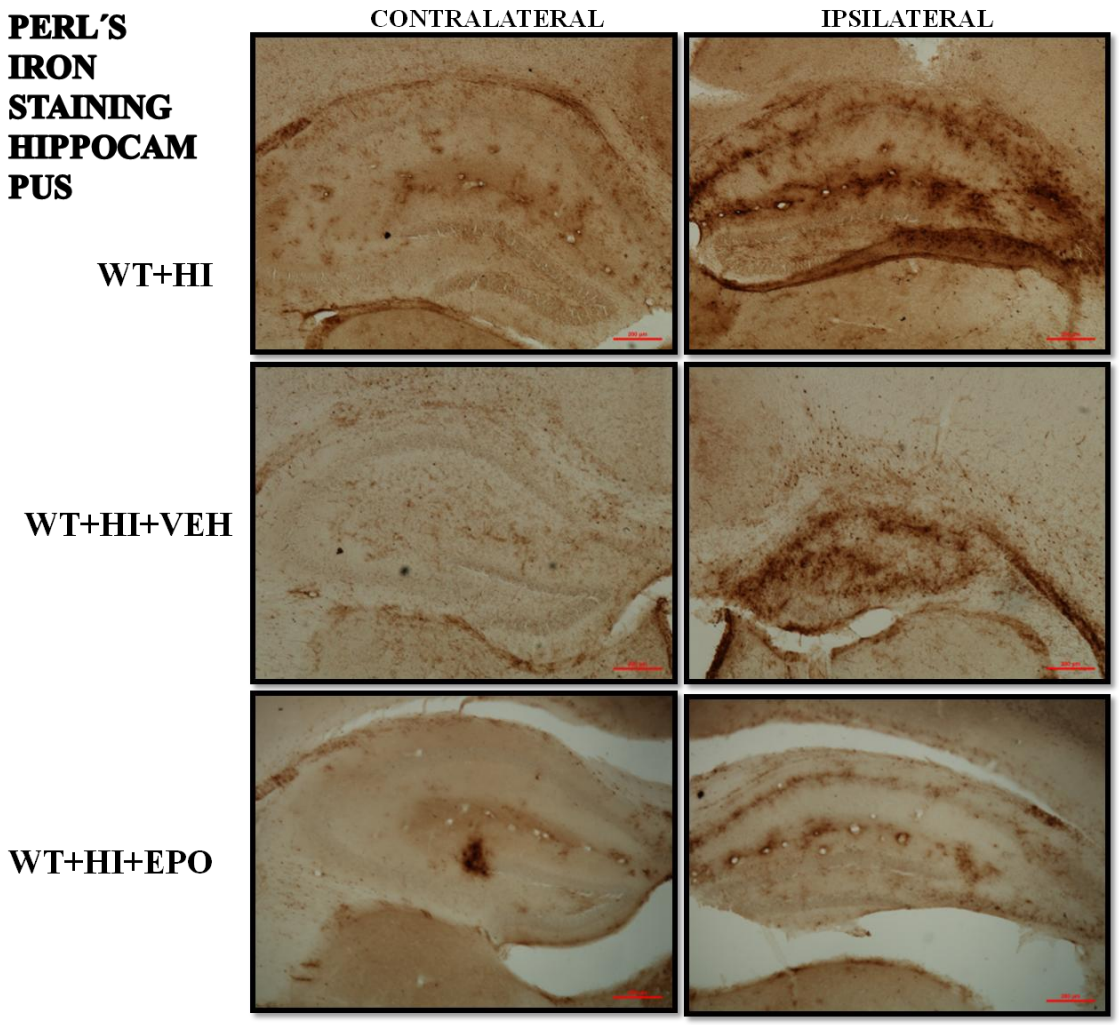
7.irudia: Nisslarekin tindaturiko burmuinaren ebakiak animalia ez-trangenikoetan (wild type), non hipokanpoko hortz zirkunbalazio (DG) zonaldeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 100 µm.

CTX

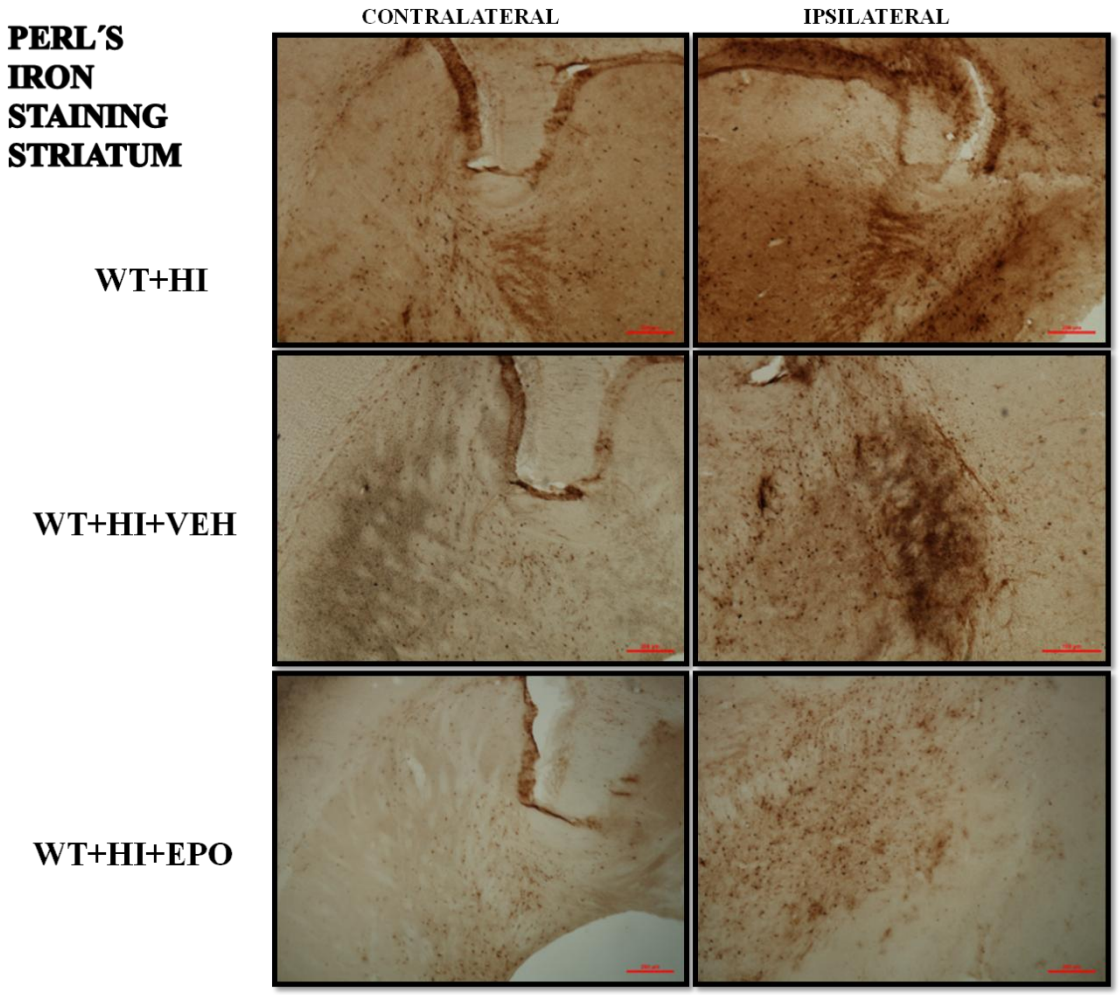


8.irudia: Nisslarekin tindaturiko burmuinaren ebakiak animalia ez-trangenikoetan (wild type), non kortexeko (CTX) zonaldeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 100 µm.

- **PERLEN BURDIN TINDAKETA**

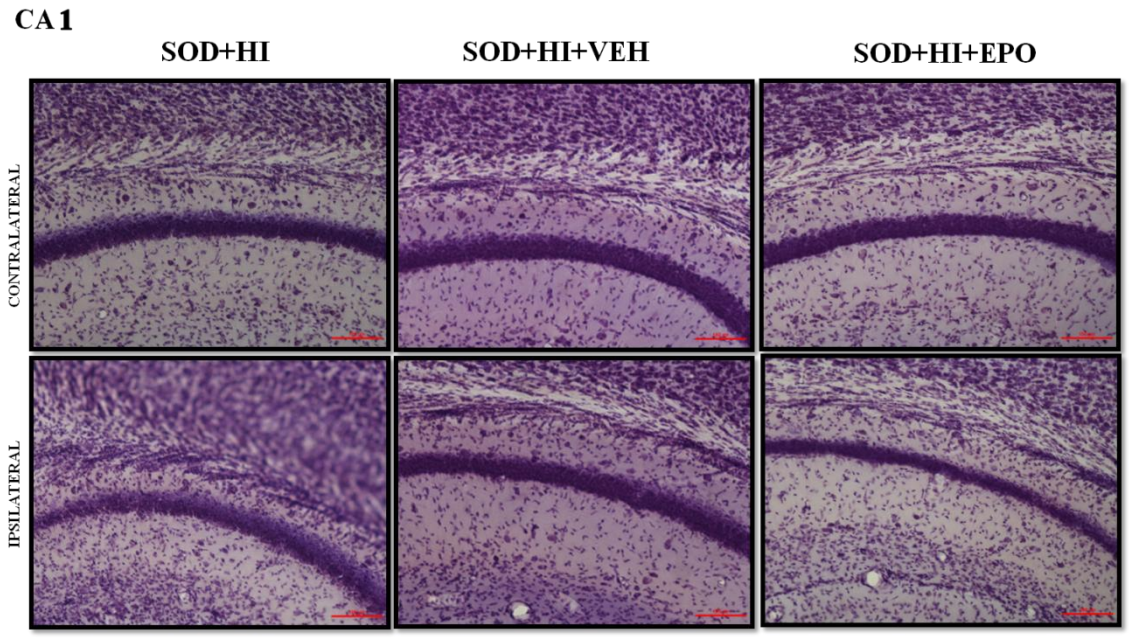


9.irudia: Perlen burdinarekin tindaturiko burmuinaren ebakiak animalia ez-trangenikoetan (wild type), non hipokanpoko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 200 µm.

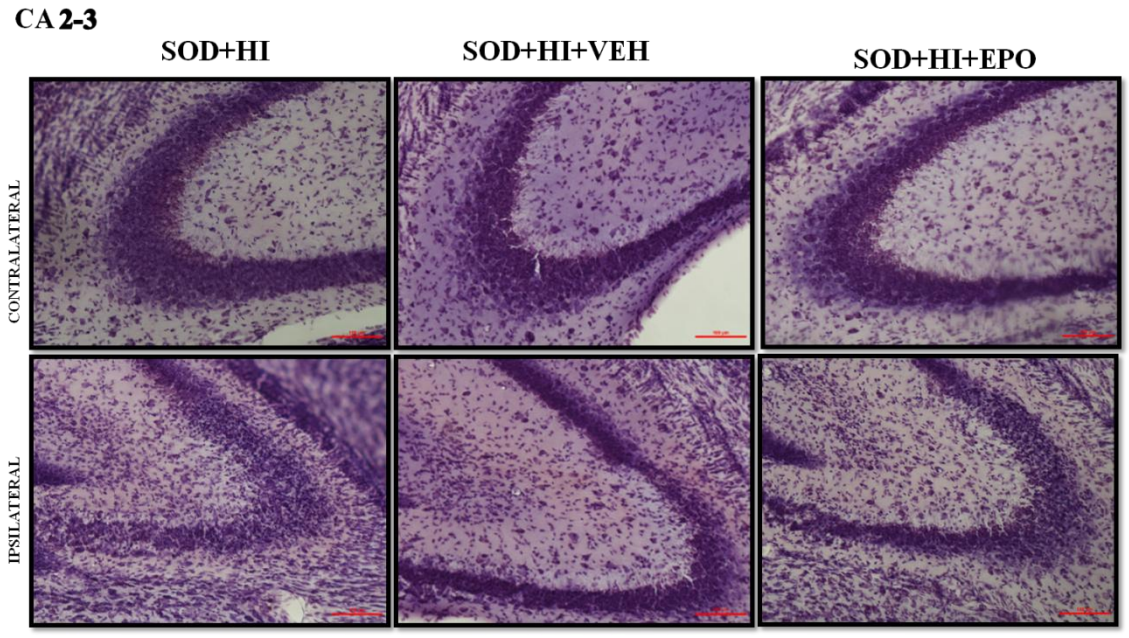


10.irudia: Perlen burdinarekin tindaturiko burmuinaren ebakiak animalia ez-trangenikoetan (wild type), non estriatumeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 200 µm.

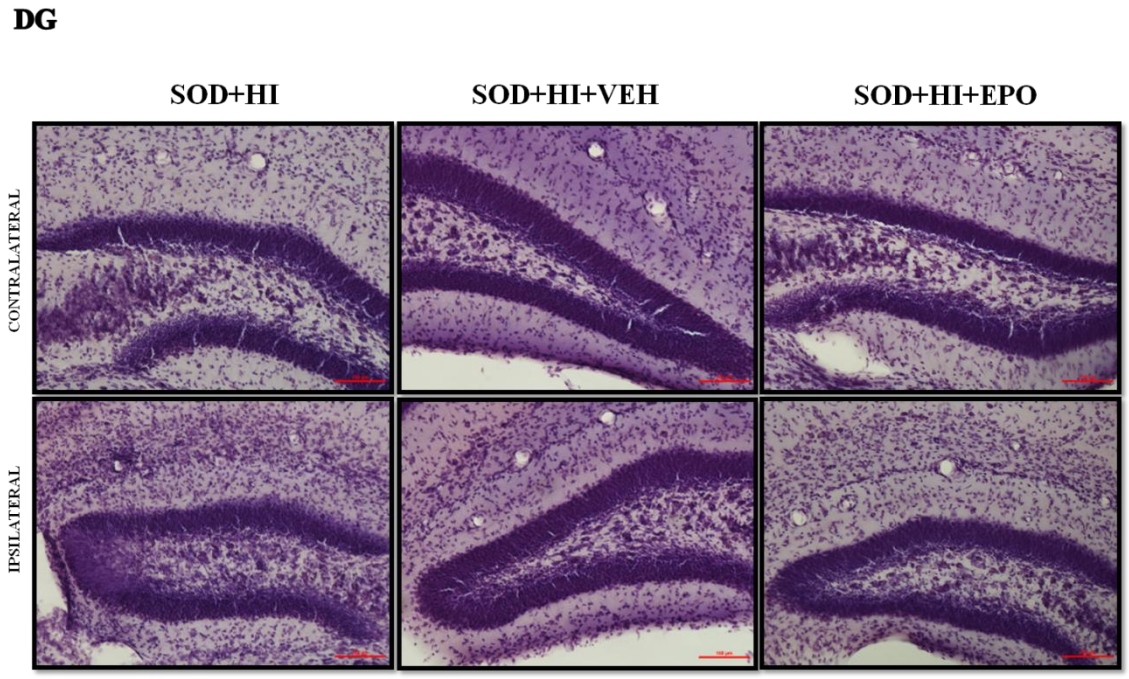
- **SOD groups (SOD entzimarako transgenikoak diren animaliak)**
  - **NISSL TINDAKETA**



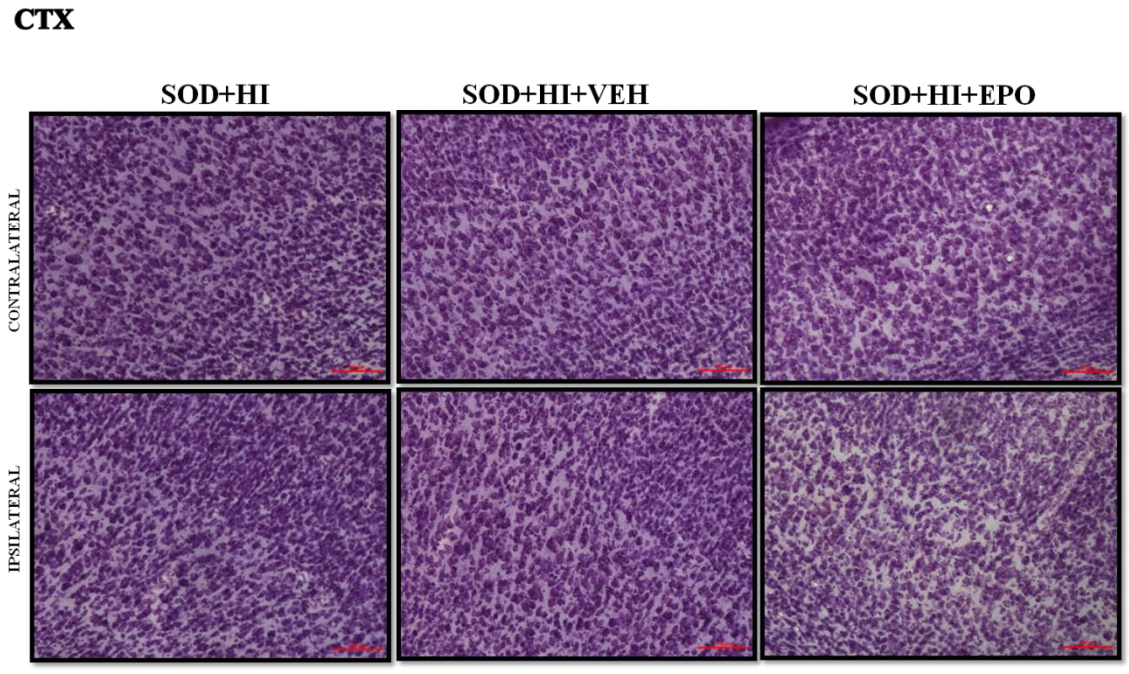
11.irudia: Nisslarekin tindaturiko burmuinaren ebakiak animalia trangenikoetan (SOD), non hipokanpoko CA 1 zonaldeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 100 µm.



12.irudia: Nisslarekin tindaturiko burmuinaren ebakiak animalia trangenikoetan (SOD), non hipokanpoko CA 2-3 zonaldeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 100 µm.

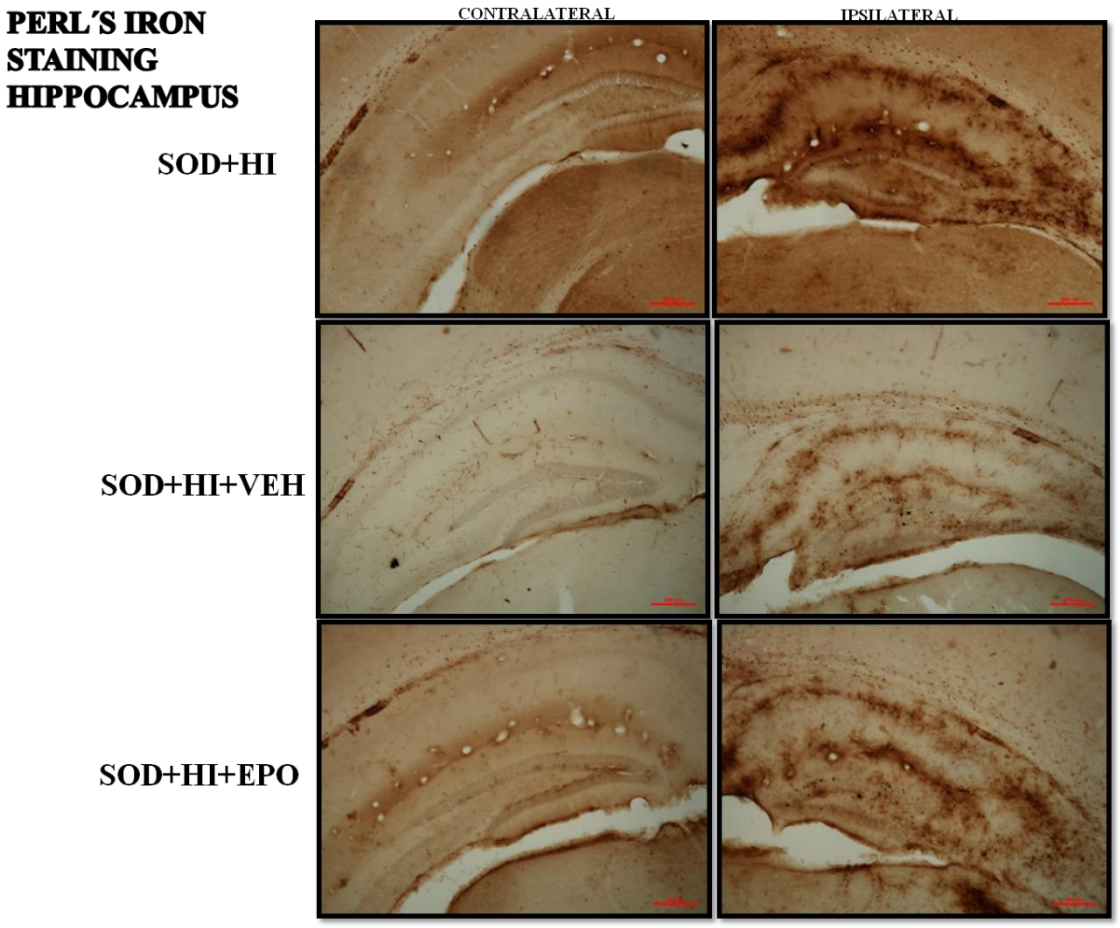


13.irudia: Nisslarekin tindaturiko burmuinaren ebakiak animalia trangenikoetan (SOD), non hipokanpoko hortz zirkunbalazio (DG) zonaldeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 100 µm.

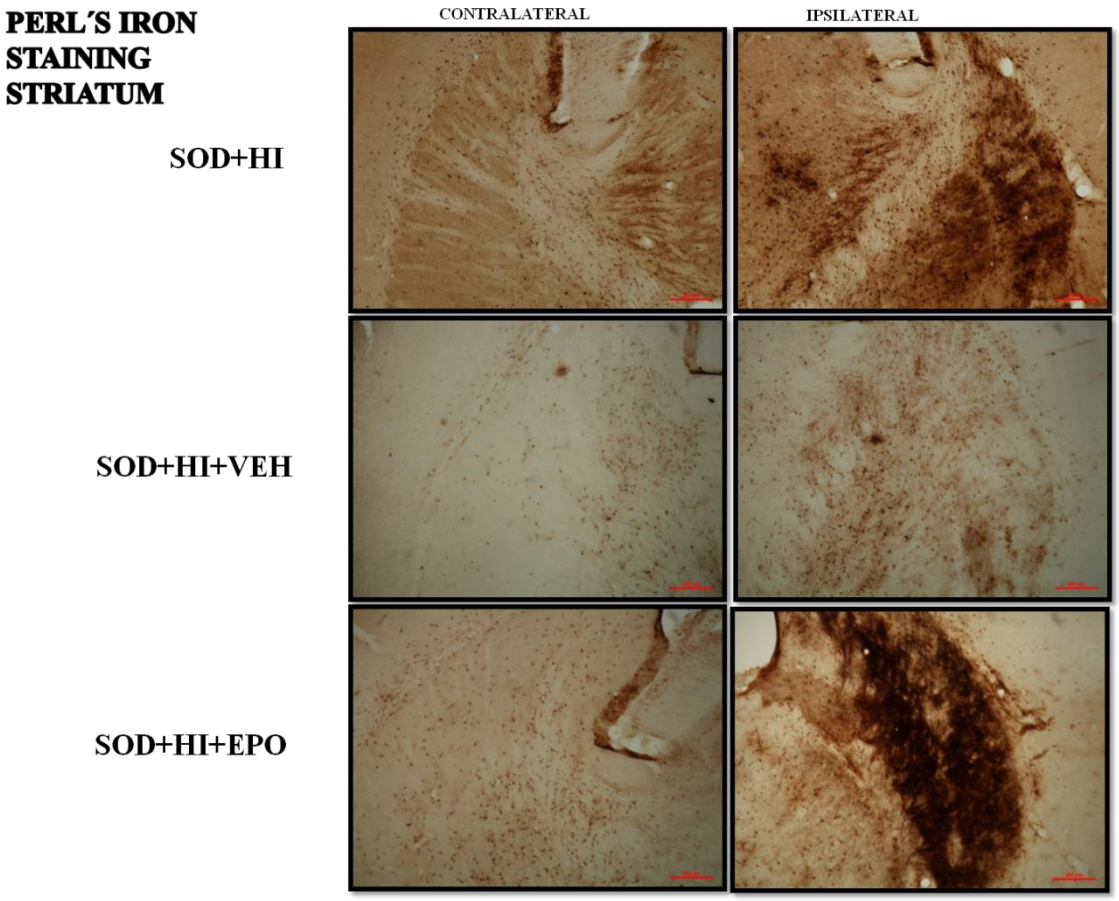


14.irudia: Nisslarekin tindaturiko burmuinaren ebakiak animalia trangenikoetan (SOD), non kortexeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 100 µm.

- **PERLEN BURDIN TINDAKETA**



15.irudia: Perlen burdinarekin tindaturiko burmuinaren ebakiak animalia trangenikoetan (SOD), non hipokanpoko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 200 µm.



16.irudia: Perlen burdinarekin tindaturiko burmuinaren ebakiak animalia trangenikoetan (SOD), non estriatumeko alde kontralteral eta ipsilaterala konparatzen diren saguek 16 egun dituztenean. Eskalaren marra: 200 µm.