Desarrollo de nanopartículas sólidas lipídicas como sistemas de administración de ADN para terapia génica

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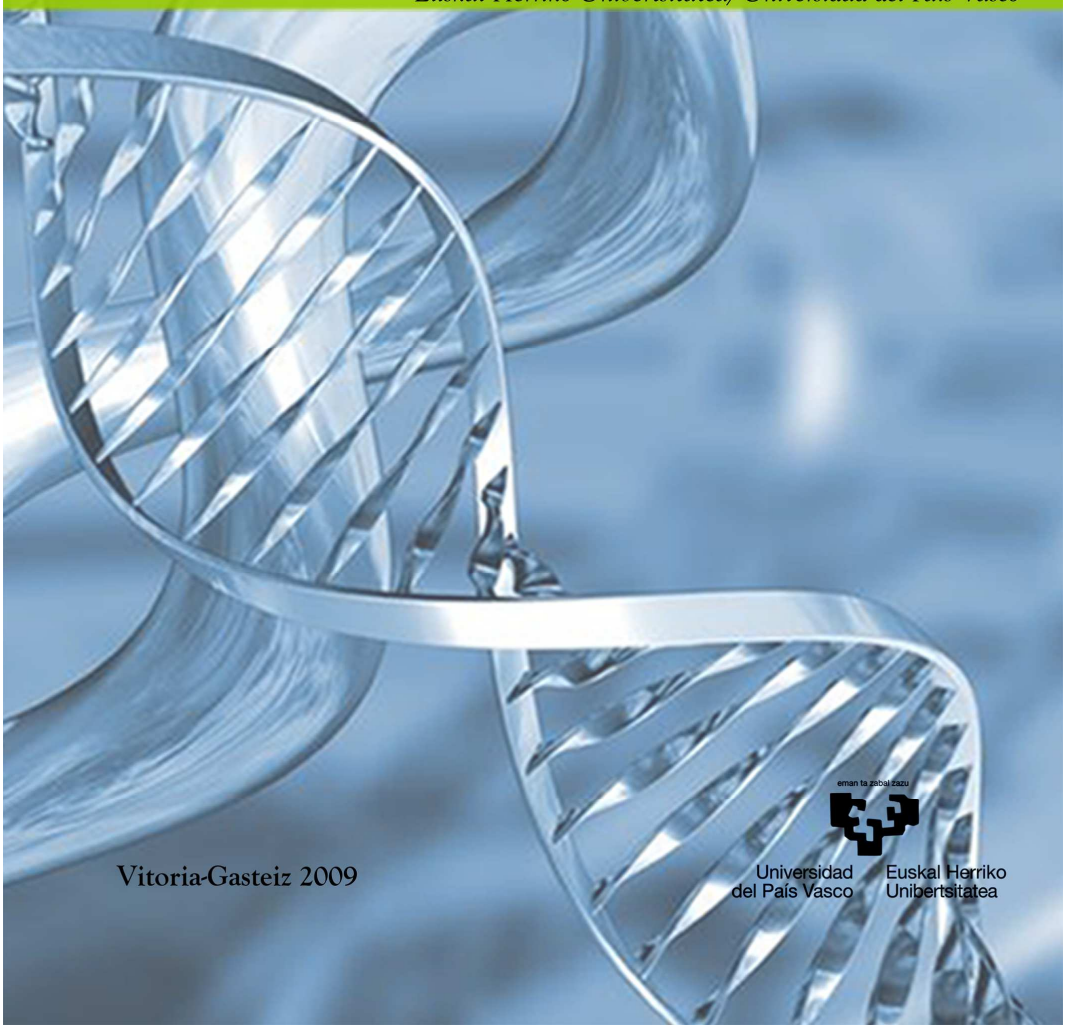

DESARROLLO DE NANOPARTÍCULAS SÓLIDAS LIPÍDICAS COMO SISTEMAS DE ADMINISTRACIÓN DE ADN PARA TERAPIA GÉNICA

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La ignorancia afirma o niega
rotundamente; la ciencia duda
Voltaire

GLOSARIO

α-1AT: α1-antitripsina
α-Gal A: α-galactosidasa A
ADA: enzima adenosin-desaminasa
ADN: ácido desoxirribonucleico
AFM: microscopía de fuerza atómica
ARN: ácido ribonucleico
ARPE-19: células humanas del epitelio pigmentario de retina
ATCC: colección de células americana
BDNF: factor neurotrofico derivado del cerebro
CAT: enzima cloranfenicol transferasa
CD: citosina desaminasa
CFTR: proteína reguladora de conductancia transmembrana
CLSM: microscopía láser confocal
CPP: péptido de penetración celular
CsCl: cloruro de cesio
dATP: desoxiATP
D-MEM: medio de cultivo Dulbecco's Modified Tagle
DNA: ácido desoxirribonucleico
DNasa: enzima desoxirribonucleasa
DNase: enzima desoxirribonucleasa
DOPE: dioleoil-fosfatidiletanolamina
DOTAP: N-[1-(2,3-Dioleoiloxi)propil]-N,N,N-trimetilamonio
EMA: monoazida de etidio
EMEM: medio de cultivo Eagle's Minimal Essential
F12: Mezcla de nutrientes de Han para medio de cultivo
GAD: descarboxilasa glutámica ácida
GAG: glucosaminoglucanos
Gb3: globotriosilceramida
GDNF: factor neurotrófico derivado de células de la glia
GFP o EGFP: proteína verde fluorescente
HEK293: células humanas de riñón embrionario
HeLa: línea celular de adenocarcinoma de cérvix

H.R.: humedad relativa
HSV-tk/GCV: sistema de la timidina quinasa del herpes simplex/ganciclovir
IFN-α2: interferón α2
IL2: interleukina 2
L-DNA o L-ADN: ADN lineal
LDV: velocimetría láser Doppler
n: número de replicados
NaCl: cloruro sódico
NGF: factor de crecimiento nervioso
NK: células natural killer
NLS: señal de localización nuclear
NPC: complejo de poros nucleares
OC-DNA u OC-ADN: ADN abierto circular
pADN: ADN plasmídico
PCS: espectroscopía de correlación fotónica
PEG: polietilenglicol
PEI: polietilenimina
pHPMA: poli(N-2-hidroxipropil)metacrilamida
PLGA: ácido poli-láctico co-glicólico
PLL: poli(L-lisina)
PVDF: polifluoruro de vinildeno
RES: sistema retículo endotelial
RGD: secuencia arginina-glicina-ácido aspártico
R.H.: humedad relativa
RNA: ácido ribonucleico
RPE: epitelio pigmentario de retina
RS1: gen de las retinosquisis juvenil ligada al sexo
SAP: Péptido "*Sweet Arrow*"
SC-DNA o SC-ADN: ADN superenrollado
SCDI: inmunodeficiencia severa combinada
SD: desviación estándar
SDS: laurilsulfato sódico
SIDA: síndrome de inmunodeficiencia humana adquirida
SLNs: nanopartículas sólidas lipídicas

SRE: sistema reticuloendotelial

Tat: trans-activador de la transcripción

T_g: temperatura de transición vítrea

VEGF: factor de crecimiento del endotelio vascular

Vif: factor de infectividad viral

VIH: virus de la inmunodeficiencia humana

VLPs: partículas similares a virus

VP22: proteasa viral del virus herpes simplex

X-SCID: inmunodeficiencia severa combinada ligada al cromosoma X

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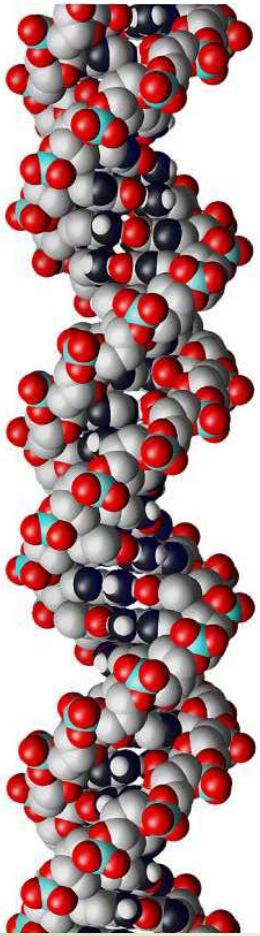

INTRODUCCIÓN

La decodificación del genoma humano y los continuos avances en biotecnología han dado lugar al desarrollo de un amplio grupo de nuevos agentes terapéuticos: los ácidos nucleicos (oligonucleótidos de pequeño tamaño, ADN o ARN). La utilización de estos agentes con fines terapéuticos constituye la terapia génica.

La terapia génica es una nueva área de la medicina con gran potencial para el tratamiento de enfermedades tanto hereditarias como adquiridas. De hecho, las enfermedades que pueden ser objeto de este tipo de terapia pueden ir desde enfermedades monogénicas hasta otras mas complicadas como el cáncer o el SIDA.

Hasta el momento, los sistemas de administración de ADN basados en virus han resultado ser los más eficaces; sin embargo, el riesgo que supone su uso ha hecho que numerosos grupos de investigación nos centremos en el desarrollo de sistemas no virales, menos eficaces pero mas seguros que los vectores virales. Para incrementar su eficacia es fundamental conocer su comportamiento dentro de la célula diana, con el fin de detectar los pasos limitantes y desarrollar estrategias que permitan superarlos.

I. CLASIFICACIÓN DE LA TERAPIA GÉNICA

La terapia génica se puede definir como el conjunto de técnicas que permiten vehiculizar fragmentos de ADN o ARN para que se expresen en el interior de células o tejidos diana, con el fin de modular la expresión de determinadas proteínas, que se encuentra alterada, revirtiendo así el trastorno biológico o enfermedad que ello produce[1]. De este modo, es posible curar las enfermedades, revirtiendo la causa de las mismas, y no solo tratar los síntomas.

En el desarrollo de la terapia génica hay que tener en cuenta la enfermedad objeto de tratamiento, la vía de administración, el gen terapéutico y el sistema de administración de ese gen. Es importante que el sistema de administración permita controlar la localización del gen en el organismo, así como el tiempo que dura su expresión.

Este tipo de terapia se puede clasificar principalmente en función de la célula diana o en función de la estrategia a seguir.

1.1. TIPOS DE TERAPIA GÉNICA EN FUNCIÓN DE LA CÉLULA DIANA

Podemos hablar de terapia génica de células germinales o terapia génica de células somáticas.

1.1.1. Terapia génica de células germinales

La terapia génica de células germinales consiste en modificar la dotación genética de las células que van a constituir los óvulos y espermatozoides, lo que permite transmitir esa modificación a la descendencia corrigiendo así enfermedades congénitas. Sin

1 Novelli G, Gruenert DC. Genome medicine: gene therapy for the millennium. Pharmacogenomics. 2002; 3: 15-18.

embargo, las importantes limitaciones éticas de la manipulación genética de células germinales han hecho que esta rama de la terapia génica no se haya desarrollado.

1.1.2. Terapia génica somática

Este tipo de terapia génica está dirigida a modificar la dotación genética de las células constituyentes del organismo, de modo que no es transmisible a la descendencia. No presenta tantas reservas éticas, y esto ha hecho que sea en este ámbito en el que se desarrollen protocolos clínicos[2].

1.2. TIPOS DE TERAPIA GÉNICA EN FUNCIÓN DE LA ESTRATEGIA A SEGUIR

A la hora de aplicar la terapia génica se puede recurrir a dos estrategias diferentes, hablándose de terapia génica “ex vivo” o terapia génica “in vivo”.

1.2.1. Terapia génica “ex vivo”

Cuando se recurre a la terapia génica “ex vivo” las células a tratar se extraen del paciente, se aíslan, se hacen crecer en cultivo y se someten al proceso de transfección “in vitro”. A continuación se seleccionan las células que han sido transfectadas eficazmente, se expanden en cultivo y se introducen de nuevo en el paciente.

Entre las ventajas de este tipo de terapia destacan la posibilidad de elegir el tipo de célula a tratar, el mayor control sobre todo el proceso y una gran eficacia de transfección.

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2 Ronchera-Oms CL, González JM. Terapia génica. En: Bonal J, Domínguez-Gil A, Gamundi MC, Napal V, Valverde E, editores. Farmacia hospitalaria. Madrid: SCM, Doyma; 2002. p. 919-927.

En cuanto a los posibles inconvenientes, hay que tener en cuenta la incapacidad de tratar tejidos cuyas células no puedan crecer en cultivo y los posibles problemas de contaminación.

1.2.2. Terapia génica “in vivo”

La terapia génica “in vivo” incluye técnicas mediante las cuales el material genético se introduce en las células del organismo sin que éstas sean extraídas del mismo y manipuladas “in vitro”.

Desde el punto de vista clínico y farmacéutico es más aceptable el uso de la terapia génica “in vivo” que la terapia génica “ex vivo”, ya que permite la utilización de las vías de administración habituales. Sin embargo, el grado de control sobre el proceso de transfección es menor, y tanto la eficiencia de transfección como el grado de especificidad tisular son bajos.

La Figura 1 recoge un esquema en el que se puede observar la diferencia entre ambos tipos de terapia.

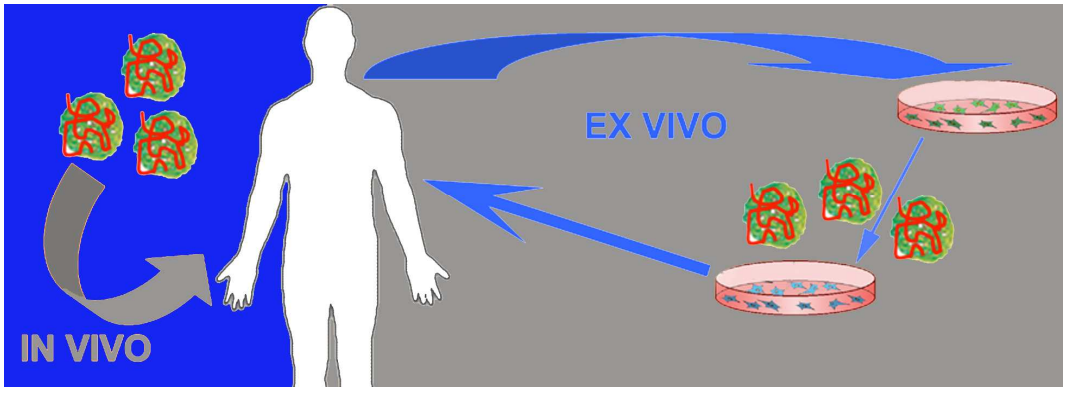

Figura 1. La terapia génica puede llevarse a cabo “in vivo” (flecha gris) o “ex vivo” (flecha azul). En la terapia génica “in vivo” el sistema de administración se introduce directamente en el organismo. En la terapia génica “ex vivo” las células que se quieren tratar se extraen del organismo, se transfectan “in vitro”, se seleccionan las que han sido transfectadas, y se vuelven a implantar en el organismo.

II. TÉCNICAS DE ADMINISTRACIÓN DE ADN

El objetivo final de la terapia génica con ADN es introducir en el núcleo celular genes de manera eficiente, sencilla y específica, de modo que las células en las que penetra ese ADN puedan sintetizar la proteína codificada por el gen correspondiente. Todo este proceso se conoce como transfección celular, y para que tenga lugar, el ADN se puede administrar utilizando diversos sistemas que se pueden clasificar en: sistemas físicos, sistemas virales y sistemas químicos. Los diferentes tipos de sistemas se encuentran resumidos en la Tabla 1.

Tabla 1. Resumen de las distintas técnicas de administración de ADN utilizadas habitualmente.

SISTEMAS FÍSICOS	SISTEMAS VIRALES	SISTEMAS QUÍMICOS
Inyección local de ADN Sistemas Libres de Agujas (*Needle-free systems*) Electroporación	Virus modificados genéticamente para que puedan expresar la proteína del gen que portan, sin ser capaces de replicarse en las células	Sistemas poliméricos Sistemas lipídicos Sistemas peptídicos Combinaciones de polímeros, lípidos o péptidos

2.1. SISTEMAS FÍSICOS

Una de las posibilidades de la terapia génica consiste en administrar ADN desnudo (*naked DNA*), es decir, administrar ADN de doble hélice que previamente se ha multiplicado en bacterias. Mediante esta técnica se ha conseguido transfección local mediante la inyección de ADN sobre todo a nivel muscular[3], pero también en hígado[4], en piel[5] y en tumores[6].

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3 Danialou G, Comtois AS, Matecki S, Nalbantoglu J, Karpati G, Gilbert R, et al. Optimization of regional intraarterial naked DNA-mediated transgene delivery to skeletal muscles in a large animal muscle. Mol Ther. 2005; 11: 257-266.

La administración intramuscular de ADN desnudo tiene gran interés en el desarrollo de vacunas basadas en terapia génica[7]. Para este fin, la administración se puede llevar a cabo mediante inyección o bien utilizando los sistemas sin agujas (*needle-free systems*). Son sistemas que contienen partículas de oro cargadas con ADN que son expulsadas mediante un gas a presión que hace que penetren en las células; cuando se utilizan para administrar ADN se conocen como pistolas de genes (*gene gun*). Dentro de este grupo, el sistema PowderJect™ XR1 (Figura 2) ha sido utilizado en ensayos clínicos en fase I como vacuna contra la hepatitis B[8] y contra el virus de la gripe[9], demostrando que las vacunas de ADN pueden inducir respuesta inmune frente a diferentes enfermedades infecciosas de manera segura.

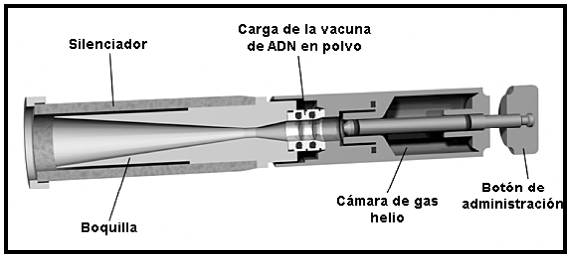

Figura 2. Esquema del sistema de administración sin agujas (*needle-free system*) PowderJect™, con el que se han llevado a cabo ensayos clínicos en Fase I.

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4 Inoue S, Hakamata Y, Kaneko M, Kobayashi E. Gene therapy for organ grafts using rapid injection of naked DNA: application to rat liver. Transplantation. 2004; 7: 997-1003.

5 Chesnoy S, Huang L. Enhanced cutaneous gene delivery following intradermal injection of naked DNA in a high ionic strength solution. Mol Ther. 2002; 5:57-62.

6 Baqué P, Pierrefite-Carle V, Gavelli A, Brossette N, Benchimol D, Bourgeon A, et al. Naked DNA injection for liver metastases treatment in rats. Hepatology. 2003; 35: 1144-1152.

7 Brown MD, Schätzlein AG, Uchegbu IF. Gene delivery with synthetic (non viral) carriers. Int J Pharm. 2001; 229: 1-21.

8 Roy MJ, Wu MS, Barr LJ, Fuller JT, Tussey LG, Speller S, et al. Induction of antigen-specific CD8-T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine. 2001; 19: 764-778.

9 Drape RJ, Macklin MD, Barr LJ, Jones S, Haynes JR, Dean HJ. Epidermal DNA vaccine for influenza is immunogenic in humans. Vaccine. 2006; 24: 4475–4481.

Otra estrategia para mejorar la eficiencia de transfección del ADN desnudo es el uso de pulsos eléctricos para permeabilizar temporalmente las membranas celulares, lo que se conoce como electroporación. Este procedimiento ha sido utilizado sobre todo como herramienta para mejorar la transfección "*in vitro*". Sin embargo, en los últimos tiempos se ha visto que resulta también efectivo "*in vivo*", principalmente para vacunas de ADN[10,11]. En este caso, tras la inyección de ADN desnudo en un tejido y la aplicación de pulsos eléctricos a nivel local se consiguen altos niveles de transfección[12].

2.2. SISTEMAS VIRALES DE ADMINISTRACIÓN DE ADN

Están compuestos por virus que han sido modificados mediante ingeniería genética de modo que permiten la expresión del gen que portan en las células diana, pero no son capaces de replicarse en ellas.

Con los sistemas virales se consigue una eficiencia de transfección muy alta, pero hay que tener en cuenta el limitado tamaño del ADN que son capaces de transportar, así como los riesgos que conlleva su uso debido a la inmunogenicidad y al potencial oncogénico.

Estos sistemas han sido evaluados en ensayos clínicos, pero la aparición de un caso de leucemia en uno de los pacientes con inmunodeficiencia severa combinada (SCDI)

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10 Scheerlinck J-PY, Karlis J, Tjelle T, Presidente PJA, Mathiesen I, Newton SE. In vivo electroporation improves immune responses to DNA vaccination in sheep. Vaccine. 2004; 22: 1820-1825.

11 Dobaño C, Widera G, Rabussay D, Doolan DL. Enhancement of antibody and cellular immune responses to malaria DNA vaccines by *in vivo* electroporation. Vaccine. 2007; 25: 6635-6645.

12 André F, Mir LM. DNA electrotransfer: its principles and an updated review of its therapeutic applications. Gene Ther. 2004; 11: S33-S42.

tratados mediante terapia génica[13] hizo que se interrumpiese el ensayo. Posteriormente, los ensayos clínicos con vectores virales se han retomado, ya que el potencial oncogénico depende de que el virus sea o no capaz de insertar el ADN que porta en el genoma de la célula huésped. Si lo inserta, se obtiene transfección a largo plazo, pero se puede inducir mutagénesis y dar lugar a células tumorales. Si no tiene lugar esa inserción el ADN queda en forma de episoma en el núcleo celular; la expresión génica es temporal, pero se evita la inducción de mutagénesis.

La inmunogenicidad es común a todos los virus y es debida a las proteínas víricas. Para el uso de los virus como sistemas de administración de ADN estas proteínas son eliminadas, con lo que se reduce en parte la capacidad inmunogénica.

Los virus más utilizados en terapia génica son los virus adeno-asociados, adenovirus, alfavirus, virus herpes simplex, retrovirus y lentivirus. La elección del virus más adecuado en cada caso dependerá de la enfermedad a tratar, y por tanto de las células que se quieren transfectar. En la Tabla 2 se han recogido las principales características de estos virus.

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13 Check E. A tragic setback. Nature. 2002; 420: 116-118.

Tabla 2. Características principales de los virus utilizados habitualmente en terapia génica.

VIRUS	CARACTERÍSTICAS
ADENO-ASOCIADOS	Transfección a largo plazo Eficiencia de transfección alta Infección de células divisibles y no divisibles
	Producción a gran escala ineficiente Capacidad de transporte de ADN limitada Integración en el genoma de la célula diana (mutagénesis)
ADENOVIRUS	Eficiencia de transfección alta Capacidad de producir grandes lotes Infección de células divisibles y no divisibles
	Inmunogenicidad Transfección transitoria
ALFAVIRUS	Inmunogenicidad baja Niveles de expresión génica muy altos
	Transfección transitoria Amplio rango de células diana Alta preferencia por las neuronas
HERPES SIMPLEX	Estado de latencia que favorece la transfeccion en células con poca división como las neuronas Menor riesgo de mutagénesis al quedar el genoma en forma de episoma Transporte de ADN de gran tamaño
	No es útil en el caso de cánceres u otras enfermedades en las que solo se requiere niveles de expresión altos pero pasajeros
RETROVIRUS	Expresión génica a largo plazo
	No es capaz de de infectar células que no se dividen Integración en el genoma de la célula
LENTIVIRUS	Infección de células que no se dividen Transporte de ADN de gran tamaño Expresión génica a largo plazo Integración en el genoma celular
	Falta de técnicas para producir a gran escala Problemas se seguridad debido a la procedencia del virus VIH

2.3. SISTEMAS QUÍMICOS DE ADMINISTRACIÓN DE ADN

Estos sistemas, a los que también se denomina de manera general sistemas no virales de administración de ADN, se obtienen mediante la multiplicación de un plásmido en bacterias que posteriormente se aísla para formularlo en un sistema de administración elaborado con diferentes componentes: polímeros, lípidos o péptidos.

Estos sistemas pueden transportar moléculas de ADN de gran tamaño, son más fáciles de producir a gran escala con bajos costes y son más seguros que los anteriores ya que no son inmunogénicos ni oncogénicos, puesto que el ADN que portan queda en forma de episoma en el núcleo de la célula.

Sin embargo, la mayor limitación que presentan es la baja eficiencia de transfección. Así como en el caso de los vectores virales se intenta buscar sistemas de administración más seguros, en el caso de los no virales muchos grupos de investigación están centrados en el desarrollo de estrategias que mejoren su capacidad de transfección.

SISTEMAS POLIMÉRICOS: En terapia génica se recurre muchas veces a los polímeros policatiónicos, que son aquellos que presentan carga positiva a pH fisiológico. De ese modo se produce una atracción electrostática entre el polímero y el ADN, que tiene carga negativa, y se forma el correspondiente complejo, llamado "*poliplex*".

Con estos componentes poliméricos se pueden elaborar tanto sistemas microparticulares como nanoparticulares. Los polímeros catiónicos más utilizados en

terapia génica son: poli (L-Lisina) (PLL)[14], polietilenimina (PEI)[15], quitosanos[16], dendrímeros[17] y ciclodextrinas[18].

También se puede recurrir a polímeros no catiónicos, como el ácido poli-láctico co-glicólico (PLGA)[19], que aunque no condensan el ADN como los polímeros catiónicos, también lo protegen de la degradación enzimática al ser incorporado dentro de las partículas. En la Figura 3 se pueden observar varias imágenes de los complejos formados mediante la unión del ADN a algunos de estos polímeros.

En ocasiones se emplean combinaciones de varios polímeros para mejorar la eficiencia de la formulación, como es el caso de la PEI con quitosanos[20] o con PLGA[21] con los que se consiguen vacunas de ADN más efectivas.

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14 Liu G, Molas M, Grossmann GA, Pasumarthy M, Perales JC, Cooper MJ, et al. Biological properties of poly-L-lysine-DNA complexes generated by cooperative binding of the polycation. J Biol Chem. 2001; 276: 34379-34387.

15 Dunlap DD, Maggi A, Soria MR, Monaco L. Nanoscopic structure of DNA condensed for gene delivery. Nucleic Acid Res. 1997; 25: 3095-3101.

16 Kim TH, Park IK, Nah JW, Choi YJ, Cho CS. Galactosylated chitosan/DNA nanoparticles prepared using water-soluble chitosan as a gene carrier. Biomaterials. 2004; 25: 3783-3792.

17 Ramaswamy C, Sakthivel T, Wilderspin AF, Florence A. Dendriplexes and their characterisation. Int J Pharm. 2003; 254: 17-21.

18 Cryan SA, Holohan A, Donohue R, Darcy R, O'Driscoll CM. Cell transfection with polycation cyclodextrin vectors. Eur J Pharm Sci. 2004; 21: 625-63.

19 Kumar R, Bakowsky U, Lehr CM. Preparation and characterization of cationic PLGA nanospheres as DNA carriers. Biomaterials. 2004; 25: 1771-1777.

20 Zhou X, Liu B, Yu X, Zha X, Zhang X, Chen Y, et al. Controlled release of PEI/DNA complexes from mannose-bearing chitosan microspheres as a potent delivery system to enhance immune response to HBV DNA vaccine. J Control Release. 2007; 121: 200-207.

21 Kasturi SP, Qin H, Thomson KS, El-Bereir S, Cha S-c, Neelapu S, et al. Prophylactic anti-tumor effects in a B cell lymphoma model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. J Control Release. 2006; 113: 261-270.

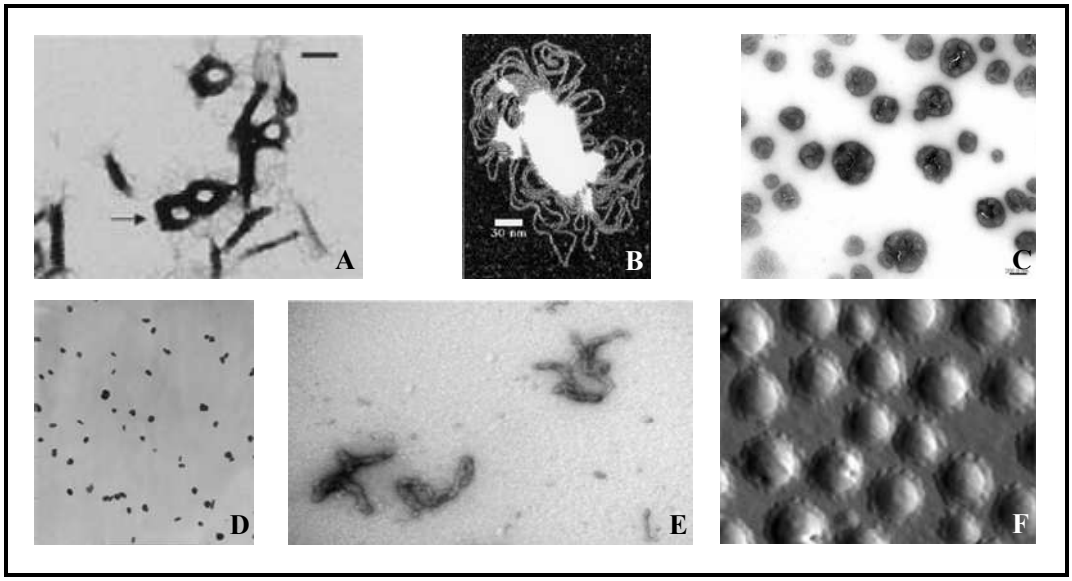

Figura 3. Fotografías de los complejos formados con diferentes polímeros utilizados en terapia génica. A: Poli-L-Lisina[14], B: polietilenimina[15], C: quitosanos[16], D: dendrímeros[17], E: ciclodextrinas[18], F: ácido poli-láctico co-glicólico[19].

Uno de los principales inconvenientes que se les atribuye a los sistemas poliméricos es la toxicidad asociada a los propios polímeros. Aunque algunos, como el PLGA, han sido aprobados para su administración en forma de micropartículas, la aplicación en sistemas nanopartículares no está aceptada. La principal razón es que a diferencia de las micropartículas, las nanopartículas poliméricas son fagocitadas por las células y la posterior degradación intracelular podría tener efectos citotóxicos[22].

SISTEMAS LIPÍDICOS: Los componentes generales de estas formulaciones son los lípidos, neutros o catiónicos. Estos últimos confieren carga positiva al sistema para que interaccione electroestáticamente con el ADN formando así el llamado "*lipoplex*".

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22 Muller RH, Keck CM. Challenges and solutions for the delivery of biotech drugs – a review of drug nanocrystal technology and lipid nanoparticles. J Biotech. 2004; 113: 151-170.

Los compuestos lipídicos son mejor tolerados por el organismo que los polímeros debido a que su estructura se asemeja más a la de los componentes fisiológicos. Aunque los lípidos catiónicos pueden inducir inflamación "*in vivo*" tras repetidas administraciones[23], ensayos clínicos llevados a cabo en pacientes con metástasis han demostrado la seguridad de vectores no virales compuestos por estos lípidos[24,25].

En el proceso de desarrollo de vectores no virales para terapia génica se utilizan fundamentalmente liposomas y nanopartículas sólidas lipídicas (SLNs), aunque también las emulsiones elaboradas con lípidos catiónicos pueden ser útiles en este tipo de terapia[26].

Los liposomas son vesículas esféricas compuestas por una o más bicapas lipídicas, que rodean un núcleo que está compuesto por un medio acuoso, mientras que las SLNs están formadas por una matriz sólida lipídica en lugar del núcleo acuoso de los liposomas. En la Figura 4 se pueden ver las diferencias estructurales entre ambos sistemas lipídicos.

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23 Han S, Mahato RI, Sung YK, Kim SW. Development of biomaterials for gene therapy. Mol Ther. 2000; 2: 302-317.

24 Galanis E, Burch PA, Richardson RL, Bradley L, Henry P, Stephen F, et al. Intratumoral administration of a 1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide/dioleoylphosphatidylethanolamine formulation of the human interleukin-2 gene in the treatment of metastatic renal cell carcinoma. Cancer. 2004; 101: 2557-2566.

25 González R, Hutchins L, Nemunaitis J, Atkins M, Schwarzenberger PO. Phase 2 trial of Allovectin-7 in advanced metastatic melanoma. Melanoma Res. 2006; 16: 521-526.

26 Kim TW, Chung H, Kwon IC, Sung HC, Jeong SY. Optimization of lipid composition in cationic emulsion as *In Vitro* and *In Vivo* transfection agents. Pharm Res. 2001; 18: 54-60.

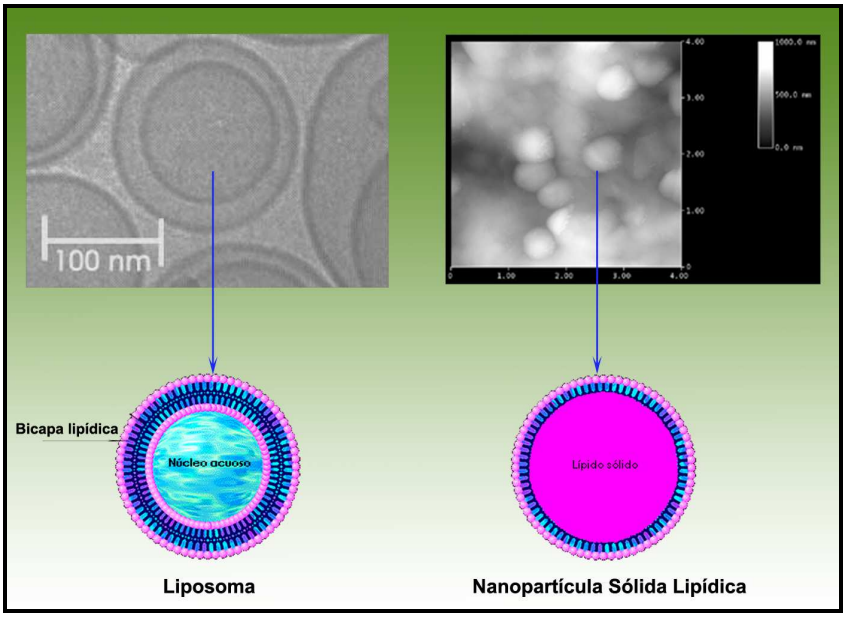

Figura 4. Diferencias estructurales entre un liposoma y una nanopartícula sólida lipídica (SLN). Los liposomas están formados por una o más bicapas lipídicas rodeando un núcleo acuoso, mientras las SLNs presentan un núcleo lipídico sólido rodeado de una capa también formada por un lípido.

En algunos casos se ha recurrido a la combinación de componentes poliméricos y lipídicos consiguiendo un efecto sinérgico[27].

SISTEMAS PEPTÍDICOS: Los péptidos catiónicos que se emplean en terapia génica contienen en su estructura aminoácidos con carga positiva, como son la histidina, la lisina o la arginina, a los que se une el ADN.

Algunos autores han propuesto la incorporación en este tipo de sistemas de péptidos sintéticos similares a secuencias activas de proteínas víricas, para incrementar la captación celular y por tanto la transfección. Uno de los primeros péptidos catiónicos

27 Lee C-H, Ni Y-H, Chen C-C, Chou C-K, Chung F-H. Synergistic effect of polyethylenimine and cationics liposomes in nucleic acid delivery to human cancer cells. Biochim Biophys Acta. 2003; 1611: 55-62.

utilizados con éxito en la transfección de cultivos celulares fue el péptido α-hélice KALA[28], que deriva de la subunidad HA-2 del virus influenza, y proporciona a ese virus la capacidad de penetrar en las células. Más recientemente también se ha propuesto el uso de los dominios de transducción de proteínas, Tat[29] o VP22[30], derivados de los virus de la inmunodeficiencia humana adquirida y herpes simplex, respectivamente, o de péptidos señal de localización nuclear (NLS) que facilitan la entrada al núcleo[31,32,33]. Sin embargo, por sí solos los sistemas peptídicos no producen suficiente transfección, por lo que hay que combinarlos con componentes lipídicos[34] o poliméricos[35].

Dentro de este grupo de sistemas de administración de ADN se pueden incluir las partículas similares a virus (virus-like particles, VLPs). Son proteínas estructurales de las cápsides virales que se reconstituyen para formar partículas similares a virus no

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28 Wyman TB, Nicol F, Zelphati O, Scaria PV, Plank C, Szoka FC. Design, synthesis and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. Biochemistry. 1997; 36: 3008-3017.

29 Ignatovich IA, Dihes EB, Pavlotskaya AV, Akifiev BN, Burov SV, Orlov SV, et al. Complexes of plasmid DNA with basic domain 47-57 of the HIV-1 Tat protein are transferred to mammalian cells by endocytosis-mediated pathways. J Biol Chem. 2003; 278: 42625-42636.

30 Suzuki K, Murtuza B, Brand NJ, Varela-Carver A, Fukushima S, Yacoub MH.Enhanced effect of myocardial gene transfection by VP22-mediated intercellular protein transport. J Mol Cell Cardiol. 2004; 36: 603-606.

31 Boulanger C, Di Giorgio C, Vierling P. Synthesis of acridine-nuclear localization signal (NLS) conjugates and evaluation of their impact on lipoplex and polyplex-based transfection. Eur J Med Chem. 2005: 40: 1295-1306.

32 Shiraishi T, Hamzavi R, Nielsen PE. Targeted delivery of plasmid DNA into the nucleus of cells via nuclear localization signal peptide conjugated to DNA intercalating Bis- and Trisacridines. Bioconjugate Chem. 2005; 16: 1112 -1116.

33 Yoo HS, Jeong SY. Nuclear targeting of non-viral gene carriers using psoralen-nuclear localization signal (NLS) conjugates. Eur J Pharm Biopharm. 2007; 66: 28-33.

34 Tokunaga M, Hazemoto N, Yotsuyanagi T. Effect of oligopeptides on gene expression: comparison of DNA/peptide and DNA/peptide/liposome complexes. Int J Pharm. 2004; 269: 71-80.

35 Lee H, Jeong JH, Park TG. A new gene delivery formulation of polyethylenimine/DNA complexes coated with PEG conjugated fusogenic peptide. J Control Release. 2001; 76: 183-192.

infectivas. Existen varios trabajos que muestran la capacidad de estas partículas para introducir ADN en células tanto "*in vitro*"[36,37] como "*in vivo*"[38,39], demostrando su potencial en terapia génica.

36 Krauzewicz N, Stokrova J, Jenkins C, Elliott M, Higgins CF, Griffin BE. Virus-like gene transfer into cells mediated by polyoma virus pseudocapsids. Gene Ther. 2000; 7: 2122–2131.

37 Gleiter S, Lilie H. Cell-type specific targeting and gene expression using a variant of polyoma VP1 virus-like particles. Biol Chem. 2003; 384: 247–255.

38 Krauzewicz N, Cos C, Soeda E, Clark B, Rayner S, Griffin BE. Sustained ex vivo and in vivo transfer of a reporter gene using polyoma virus pseudocapsids. Gene Ther. 2000; 7: 1094–1102.

39 Tegerstedt K, Andreasson K, Vlastos A, Hedlund KO, Dalianis T, Ramqvist T. Murine pneumotropic virus VP1 virus-like particles (VLPs) bind to several cell types independent of sialic acid residues and do not serologically cross react with murine polyomavirus VP1 VLPs. J Gen Virol. 2003; 84: 3443–3452.

III. TRANSFERENCIA GÉNICA CON SISTEMAS NO VIRALES

El proceso por el cual el ADN exógeno penetra en las células para ser finalmente introducido en el núcleo y desencadenar la síntesis de la proteína que codifica se conoce como transferencia génica o transfección.

Las principales etapas que debe superar el material genético para llegar al núcleo se exponen a continuación y se resumen en la Figura 5:

- Fijación a la superficie celular.
- Entrada en el citoplasma.
- Escape endosomal del ADN al citoplasma.
- Entrada del ADN en el núcleo.

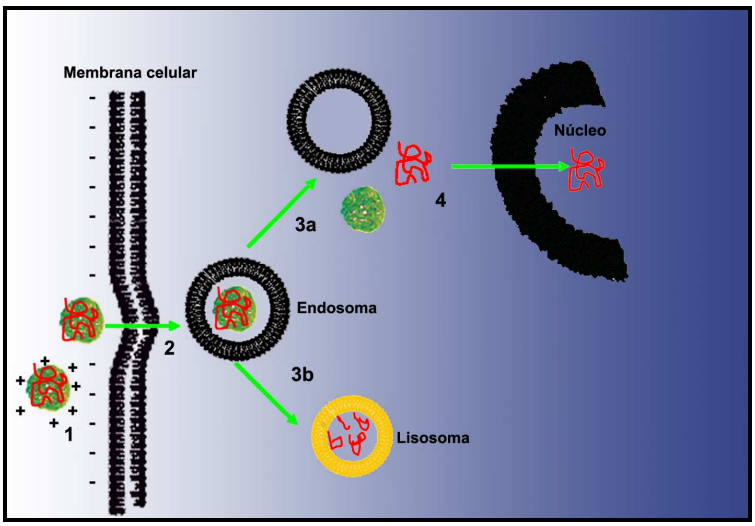

Figura 5. Esquema de las etapas que debe superar el ADN para pasar del medio extracelular al núcleo de la célula. En primer lugar el sistema de administración debe ser capaz de unirse a la membrana de las células diana (1). Una estrategia muy utilizada para ello es el uso de sistemas catiónicos que mediante interacciones electrostáticas se unen a cargas negativas de la membrana celular. Posteriormente, tendrá lugar la entrada a la célula mayoritariamente mediante endocitosis (2). El endosoma debe liberar el ADN (3a) antes de fundirse con los lisosomas (3b), cuyas enzimas pueden hidrolizarlo. Una vez que el ADN se encuentra en el citoplasma celular tiene que penetrar en el núcleo de la célula atravesando la membrana nuclear (4).

La mayor seguridad de los vectores no virales ha dado lugar a que muchos grupos de investigación estén centrados en mejorar su eficacia, es decir, su capacidad de

transfección. Las estrategias a seguir se centran en facilitar cada una de las etapas que el ADN debe superar para penetrar finalmente en el núcleo, por lo que es fundamental conocer con la mayor precisión posible el proceso completo de transfección.

3.1 DISTRIBUCIÓN DEL ADN EN EL ORGANISMO

La aplicación "*in vivo*" de la terapia génica requiere como primer paso que el gen terapéutico llegue a las célula diana. La distribución del ADN en el organismo va a estar condicionada por la vía y el sistema de administración, y es la principal responsable de la falta de correlación observada entre la transfección obtenida "*in vitro*" e "*in vivo*". Una vez administrado el ADN, la respuesta inmune y la interacción con los distintos componentes del organismo son los dos factores que mayor influencia van a tener en la eficacia y seguridad de los sistemas de administración de ADN. Se debe conseguir que todo el ADN llegue específicamente a las células diana, que sea capaz de transfectar, y que la transfección sea segura y eficaz.

Los sistemas no virales, en principio, no van a tener el problema de la inmunogenicidad que se atribuye a los sistemas virales, aunque la mayoría de las partículas administradas poseen cargas superficiales y por tanto pueden activar el sistema del complemento[40], siendo en este caso eliminadas del torrente sanguíneo al ser captados por macrófagos del sistema reticuloendotelial. En cualquier caso, esa

40 Chonn A, Cullis PR, Devine DV. The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. J Immunol. 1991; 146: 4234-4241.

capacidad de activación no depende únicamente de la carga superficial sino que también influye la composición del sistema administrado[41].

Como los sistemas no virales de ADN son captados por las células en general de manera inespecífica, su aplicación "*in vivo*" se limita mayoritariamente a la administración local. A través de esta vía se ha conseguido transfección administrando tanto ADN desnudo, como incorporado en vectores virales y no virales en tumores[42,43,44], pulmones[45,46], cerebro[47,48], corazón[49], riñones[50], hígado[51,52], retina[53] y músculo[54,55,56,57].

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41 Plank C, Mechtler K, Szoka FC, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. Hum Gene Ther. 1996; 7: 1437-1446.

42 Brown MD, Schätzlein AG, Uchegbu IF. Gene delivery with synthetic (non viral) carriers. Int J Pharm. 2001; 229: 1-21.

43 Yockman JW, Maheshwari A, Han S, Kim SW. Tumor regression by repeated intratumoral delivery of water soluble lipopolymers/p2CMVmIL-12 complexes. J Control Release. 2003; 87: 177-186.

44 Carri NG, Sosa YE, Brown OA, Albariño C, Romanowski V, Goya RG. Studies on in vivo gene transfer in pituitary tumors using herpes-derived and adenoviral vectors. Brain Res Bull. 2005; 65:17-22.

45 Gautam A, Densmore CL, Golunski E, Xu B, Waldrep JC. Transgene expression in mouse airway epithelium by aerosol gene therapy with PEI-DNA complexes. Mol Ther. 2001; 3: 551-556.

46 Price A, Limberis M, Gruneich JA, Wilson JM, Diamond SL. Targeting viral-mediated transduction to the lung airway epithelium with the anti-inflammatory cationic lipid dexamethasone-spermine. Mol Ther. 2005; 12: 502-509.

47 Leone P, Janson GC, Bilianuk L, Wang Z, Sorgi F, Huang L, et al. Aspartoacylase gene transfer to the mammalian central nervous system with therapeutic implications for Canavan disease. Ann Neurol. 2001; 48: 27-38.

48 Li H-W, Gao Y-x, Matsuura T, Martynyuk A, Raizada MK, Summers C. Adenoviral-mediated neuron specific transduction of angiotensin II type 2 receptors. Regul Pept. 2005; 126: 213-222.

49 Bull DA, Bailey SH, Rentz JJ, Zebrack JS, Lee M, Litwin SE, et al. Effect of Terplex/VEGF-165 gene therapy on left ventricular function and structure following myocardial infarction VEGF gene therapy for myocardial infarction. J Control Release. 2003; 93: 175-181.

50 Tsujie M, Isaka Y, Ando Y, Akagi Y, Kaneda Y, Ueda N, et al. Gene transfer targeting interstitial fibroblasts by the artificial viral envelope-type hemagglutinating virus of Japan liposome method. Kidney Int. 2000; 57: 1973.

La eficacia y complejidad de la administración van a depender fundamentalmente del órgano o tejido a tratar. En la Tabla 3 se describen las limitaciones que presenta la administración local de ADN en algunos tejidos.

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51 Baqué P, Pierrefite-Carle V, Gavelli A, Brossette N, Benchimol D, Bourgeon A, et al. Naked DNA injection for liver metastases treatment in rats. Hepatology. 2003; 35: 1144-1152.

52 Hodges BL, Taylor KM, Chu Q, Scull SE, Serriello RG, Anderson SC, et al. Local delivery of a viral vector mitigates neutralization by antiviral antibodies and results in efficient transduction of rabbit liver. Mol Ther. 2005; 12: 1043-1051.

53 Acland GM, Aguirre GD, Bennett J, Aleman TS, Cideciyan AV, Bennicelli J, et al. Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. Mol Ther. 2005; 12: 1072-1082.

54 Zhang G, Budker V, Williams P, Subbotin V, Wolff JA. Efficient expression of naked DNA delivered intraarterially to limb muscles of nonhuman primates. Hum Gene Ther. 2001; 12: 427:438.

55 Shyu KG, Wang MT, Wang BW, Chang CC, Leu JG, Kuan P, et al. Intramyocardial injection of naked DNA encoding HIF-1a/VP16 hybrid to enhance angiogenesis in an acute myocardial infarction model in the rat. Cardiovascular Res. 2002; 54: 576-583.

56 Schek RM, Hollister SJ, Krebsbach PH. Delivery and protection of adenoviruses using biocompatible hydrogels for localized gene therapy. Mol Ther. 2004; 9: 130-138.

57 Danialou G, Comtois AS, Matecki S, Nalbantoglu J, Karpati G, Gilbert R, et al. Optimization of regional intraarterial naked DNA-mediated transgene delivery to skeletal muscles in a large animal muscle. Mol Ther. 2005; 11: 257-266.

Tabla 3. Limitaciones que presentan algunos tejidos para la administración local de la terapia génica.

ÓRGANO	VÍA DE ADMINISTRACIÓN	OBSTÁCULOS	REFERENCIA
PULMONES	Aplicación directa a través de las vías respiratorias	Mucosidad en la superficie celular que se ve incrementada en procesos infecciosos Obstaculización de la captación de estructuras particuladas debido a la estructura epitelial Fagocitosis por parte de los macrófagos alveolares	**58**
TUMORES	Administración a través de las arterias que irrigan el tumor Administración directa en el tejido tumoral (administración intratumoral)	La distribución a través del tejido tumoral es pobre siendo limitada la eficiencia de transfección en todo el tumor Dificultad de los complejos para difundir por el tejido tumoral	**58**
CEREBRO	Administración a través de la arteria carótida interna. Administración directa en estructuras específicas del cerebro	La distribución depende de la movilidad y la estabilidad de los sistemas de administración	**58,** 59

La administración sistémica incluye tanto la vía oral como las vías parenterales. La vía oral se puede utilizar para corregir un defecto, local o sistémico, y también cuando se busca inmunización a nivel de las mucosas a través de las placas de Peyer. Las principales ventajas de esta vía son la posibilidad de obtener un efecto local o sistémico y la facilidad de administración, lo que supondrá una mejora en el cumplimiento

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58 Merdan T, Kopecek J, Kissel T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. Adv Drug Deliv Rev. 2002; 54: 715-758.

59 Goula D, Remy JS, Erbacher P, Wasowicz M, Levi G, Abdallah B, et al. Size, diffusibility and transfection performance of linear PEI/DNA complexes in the mouse central nervous system. Gene Ther. 1998; 5: 712-717.

terapéutico por parte del paciente. Sin embargo, no se pueden ignorar las limitaciones de esta vía de administración: el pH del estómago, que puede alterar la estructura del ADN, la presencia de nucleasas, peptidasas y lipasas en el tracto gastrointestinal y la baja permeabilidad del epitelio intestinal a los genes y sus sistemas de administración. La Tabla 4 recoge algunas estrategias utilizadas para superar los problemas que presenta esta vía tras la administración tanto de vectores virales como no virales.

Tabla 4. Estrategias que pueden mejorar la transfección llevando a cabo administración por vía oral

ESTRATEGIAS	MECANISMO	REF.
Incorporar simultáneamente lentinan	Adyuvante de la respuesta celular	**60**
Uso de quitosanos N-acetilados	Resistencia al medio ácido	**61**
Modificación con la adesina σ1 del reovirus	Vectorización hacia las células M de las placas de Peyer, que expresan el receptor para esa proteína	**62**
Modificación con invasinas de *Yersinia*	Vectorización hacia las células M de las placas de Peyer, que expresan las integrinas β1	**63**
Modificación con la subunidad B de la toxina del cólera	Vectorización hacia las células que contengan el ganglíosido G_{M1}, como las células M de las placas de Peyer	**64**
Modificación con lectinas	Vectorización hacia células M y enterocitos Bioadhesión a través de glicoproteínas	**65**
Pretratamiento con agentes mucolíticos: N-acetil cisteína	Fluidificación de la capa mucosa del tracto gastrointestinal, que dificulta la absorción	**66**

Dentro de las vías parenterales, la más utilizada es la intravenosa. Sin embargo, utilizando ADN desnudo sólo se consiguen niveles interesantes de transfección cuando

se inyectan volúmenes grandes a velocidades altas[60]. De ahí la importancia de formular el ADN para conseguir niveles adecuados de transfección mediante la administración intravenosa habitual. El tamaño del sistema de administración y la permeabilidad del endotelio vascular son determinantes para conseguir una terapia adecuada. Se debe tener en cuenta que el endotelio de órganos como el hígado, el bazo, la médula ósea y muchos tumores, presenta poros a través de los cuales pueden pasar moléculas de entre 0,1 y 1 μm, pero en el resto de órganos y tejidos el acceso a sus células puede verse limitado porque los sistemas de administración no son capaces de salir del sistema circulatorio. Por otro lado, los sistemas no virales pueden interaccionar con componentes sanguíneos formando agregados que quedan retenidos en el lecho capilar de los pulmones. Se han documentado casos en los que tras la administración intravenosa, una alta proporción del ADN administrado se acumula en el tejido pulmonar[61, 62]. Aunque este hecho puede tener interés en el tratamiento de enfermedades pulmonares como la fibrosis quística o el cáncer de pulmón, limita el uso "*in vivo*" en otro tipo de enfermedades. Además, la unión con otros componentes, como los eritrocitos, puede dar lugar a embolias pulmonares debidas a la obstrucción de vasos sanguíneos. La Tabla 5 recoge diferentes estrategias a seguir para disminuir o evitar la interacción de los sistemas no virales con los componentes sanguíneos.

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60 Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 1999; 6:1258-1266.

61 Verbaan FJ, Oussoren C, van Dam IM, Takakura Y, Hashida M, Crommelin DJ, et al. The fate of poly(2-dimethyl amino ethyl)methacrylate-based polyplexes after intravenous administration. Int J Pharm. 2001; 214: 99-101.

62 Chollet P, Favrot MC, Hurbin A, Coll JL. Side-effects of a systemic injection of linear polyethilenimine-DNA complexes. J Gene Med. 2002; 4: 84-91.

Tabla 5. Posibles estrategias para mejorar la distribución sistémica de los vectores no virales.

ESTRATEGIA	MECANISMO	REFERENCIA
Adición de cadenas de polietilenglicol (PEG)	Genera sistemas de administración cercanos a la neutralidad ↓ Disminuye las interacciones con componentes sanguíneos	**58**
Incorporación de poli(N-2-hidroxipropil)metacrilamida (pHPMA)	Neutraliza la carga superficial ↓ Disminuye las interacciones con la albúmina y reduce la fagocitosis por parte de los macrófagos "*in vitro*"	**58**
Adición de transferrina	Neutraliza la carga superficial ↓ Disminuye las interacciones con los eritrocitos	**63**

3.2 FIJACIÓN A LA SUPERFICIE CELULAR

Una vez que el ADN ha llegado al órgano de destino debe entrar en contacto con las células diana.

La parte externa de la membrana celular está cargada negativamente, por lo que, en ausencia de un ligando específico, los sistemas de administración con carga superficial positiva se unen a las células a través de fuerzas electrostáticas[64].

63 Kircheis R, Wightman L, Scheiber A, Robitza B, Rossler V, Kursa M, et al. Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. Gene Ther. 2001; 8: 28-40.

64 Pires P, Simoes S, Nir S, Gaspar R, Düzgünes N, Pedroso de Lima MC. Interaction of cationic liposomes and their DNA complexes with monocytic leucemia cells. Biochim Biophys Acta. 1999; 1418: 71-84.

Algunos de los responsables de la carga negativa de la superficie celular son los glucosaminoglucanos (GAGs). Se trata de polisacáridos aniónicos cuya carga negativa puede ser debida a grupos sulfato (heparán sulfato o condroitín sulfato) o a grupos carboxílicos (ácido hialurónico). Cuando se unen a proteínas, forman los proteoglicanos. Estos GAGs se encuentran también en la matriz extracelular, y su interacción con los sistemas de administración a este nivel puede afectar a la transfección de diferentes maneras, dificultando su llegada a las células a las que se dirige o provocando la liberación del ADN de los sistemas de administración antes de entrar en la célula. La interacción entre los sistemas de administración de ADN y los GAGs y su efecto sobre la transfección van a depender de la estructura del GAG y de la composición del sistema de administración. Ruponen y col.[65], por ejemplo, han demostrado que también los GAGs de la superficie celular inhiben la transfección de sistemas de administración de ADN catiónicos, tanto lipídicos como poliméricos. Aunque los GAGs de la membrana celular actúan como receptores de los vectores catiónicos debido a su carga negativa, parece ser que posteriormente conducen estos sistemas hacia compartimentos intracelulares en los que no se puede llevar a cabo la transcripción. Por lo tanto, el hecho de que los sistemas tengan carga positiva no asegura la fijación a la superficie celular, ni el posterior proceso de transcripción y traducción de la proteína correspondiente.

Con el fin de facilitar la unión a la superficie celular, incrementando además la selectividad, se pueden utilizar ligandos que se unan de manera específica a receptores de determinadas células. En la Tabla 6 se recogen algunos ejemplos de estos ligandos.

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65 Ruponen M, Honkakoski P, Tammi M, Urtti A. Cell-surface glycosaminoglycans inhibit cation-mediated gene transfer. J Gene Med. 2004; 6: 405–414.

Tabla 6. Ejemplos de ligandos que permiten vehiculizar el gen a unas determinadas células y pueden facilitar la unión a la superficie celular.

LIGANDO	ÓRGANO DIANA	REFERENCIA
Secuencias RGD (Arginina-Glicina- Ácido aspártico)	Células endoteliales de tumores Neuronas	**73, 74**
Transferrina	Células tumorales (con gran capacidad de proliferación)	**75**
Lactoferrina	Cerebro	**76**
Ácido fólico	Células tumorales	**77**
Azúcares	Hígado	**78**

66 Temming K, Schiffelers RM, Molema G, Kok RJ. RGD-based strategies for selective delivery of therapeutics and imaging agents to the tumour vasculature. Drug Resist Update. 2005; 8: 381-402.

67 Suk JS, Suh J, Choy K, Lai SK, Fu J, Hanes J. Gene delivery to differentiated neurotypic cells with RGD and HIV Tat peptide functionalized polymeric nanoparticles. Biomaterials. 2008; 27: 5143-5150.

68 Neves SS, Sarmento-Ribeiro AB, Simoes SP, Pedroso de Lima MC. Transfection of oral cancer cells mediated by transferrin-associated lipoplexes: Mechanisms of cell death induced by herpes simplex virus thymidine kinase/ganciclovir therapy. Biochim Biophys Acta. 2006; 1758: 1703-1712.

69 Huang R, Ke W, Liu Y, Jiang C, Pei Y. The use of lactoferrin as a ligand for targeting the polyamidoamine-based gene delivery system to the brain. Biomaterials. 2008; 29: 238-246.

70 Liang B, He M-L, Xiao Z-P, Li Y, Chan C-Y, Kung HF et al. Synthesis and characterization of folate-PEG-grafted-hyperbranched-PEI for tumor-targeted gene delivery. Biochem Biophys Res Commun. 2008; 367: 874-880.

71 Hashida M, Nishikawa M, Yamashita F, Takakura Y. Cell-specific delivery of genes with glycosylated carriers. Adv Drug Deliv Rev. 2001; 52: 187-196.

Otra ventaja de utilizar estos factores es el hecho de que al vehiculizar el gen a unas determinadas células se pueden evitar efectos adversos derivados del acceso a otras células.

3.3 ENTRADA EN EL CITOPLASMA

Una vez que el complejo se ha unido a la superficie celular debe entrar en el citoplasma. En general se considera que la principal vía de entrada en las células es la endocitosis[72,73,74,75,76], habiéndose descrito distintos tipos de entrada a través de endosomas que incluyen fagocitosis, macropinocitosis, entrada a través de clatrinas o endocitosis mediada por caveolas. Además, la fusión con la membrana celular también puede contribuir al proceso de internalización celular[77]. En función del tipo de célula, de la composición, el tamaño y la carga superficial del sistema de administración, puede predominar una u otra vía de entrada. Por ejemplo, parece que la carga positiva de los sistemas de administración facilita la invaginación de la membrana celular iniciando así

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72 Zhou X, Huang L. DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. Biochim Biophys Acta. 1994; 1189: 195-203.

73 Xu Y, Szoka FC Jr. Mechanism of DNA release form cationic liposome/DNA complexes used in cell transfection. Biochemistry. 1996; 35: 5616-5623.

74 Hafez IM, Maurer N, Cullis PR. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther. 2001; 8: 1188-1196.

75 Zuhorn IS, Kalicharan R, Hoekstra D. Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. J Biol Chem. 2002; 277: 18021-18028.

76 Rejman J, Conese M, Hoekstra D. Gene transfer by means of lipo- and polyplexes: role of clathrin and caveolae-mediated endocytosis. J Lipos Res. 2006; 16: 237-247.

77 Zhang Y, Qi XR, Gao Y, Wei L, Maitani Y, Nagai T. Mechanisms of co-modified liver-targeting liposomes as gene delivery carriers based on cellular uptake and antigens inhibition effect. J Control Release. 2007; 117: 281-290.

el proceso de endocitosis[78]. Cuando intervienen procesos de fusión, puede liberarse ADN al medio extracelular en lugar de al citoplasma[79].

Dada la importancia que parece tener la endocitosis, la adición de ligandos dirigidos a receptores que faciliten este proceso puede ser interesante para mejorar la transfección de los sistemas no virales. El uso de sistemas galactosilados, por ejemplo, mejora la eficiencia de transfección en los hepatocitos a través de la unión a las asialoglicoproteínas[80,81], que son proteínas de membrana. También se puede recurrir al uso de los péptidos de penetración celular (*cell penetrating peptides* o CPP), que a través de procesos endocíticos facilitan que las macromoléculas como el ADN entren en las células[82,83,84,85].

En cualquier caso, la unión a la superficie celular y la entrada de los complejos al citoplasma no tienen por qué ser los pasos limitantes del proceso de transfección. Es

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78 Elouahabi A, Ruysschaert JM. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol Ther. 2005; 11: 336–347.

79 Pedroso de Lima MC, Simoes S, Pires P, Faneca H, Düzgünes N. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. Adv Drug Deliv Rev. 2001; 47: 277-294.

80 Cook SE, Park IK, Kim EM, Jeong HJ, Park TG, Choi YJ, et al. Galactosylated polyethylenimine-graft-poly(vinyl pyrrolidone) as a hepatocyte-targeting gene carrier. J Control Release. 2005; 105: 151-163.

81 Zhang X-Q, Wang X-L, Zhang P-C, Liu Z-L, Zhuo R-X, Mao H-Q et al. Galactosylated ternary DNA/polyphosphoramidate nanoparticles mediate high gene transfection efficiency in hepatocytes. J Control Release. 2005; 102: 749-763.

82 Hyndman L, Lemoine JL, Huang L, Porteous DJ, Boyd AC, Nan XS. HIV-1 Tat protein transduction domain peptide facilitates gene transfer in combination with cationic liposomes, J Control Release. 2004; 99:435-444.

83 Christiaens B, Dubruel P, Grooten J, Goethals M, Vandekerckhove J, Schacht E, et al. Enhancement of polymethacrylate-mediated gene delivery by Penetratin. Eur J Pharm Sci. 2005; 24: 525-537.

84 Suzuki K, Murtuza B, Brand NJ, Varela-Carver A, Fukushima S, Yacoub MH. Enhanced effect of myocardial gene transfection by VP22-mediated intercellular protein transport. J Mol Cell Cardiol. 2004; 36: 603-606.

85 Pujals S, Fernandez-Carneado J, Ludevid D, Giralt E. D-SAP: a new, non-cytotoxic and fully protease resistant cell-penetrating peptide. ChemMedChem. 2008; 3: 296-301.

necesario tener en cuenta otros procesos, como la liberación del ADN al citoplasma y su posterior entrada al núcleo.

3.4 ESCAPE ENDOSOMAL DEL ADN AL CITOPLASMA

Como se ha comentado en el apartado anterior la vía de entrada va a condicionar el posterior comportamiento del sistema de administración dentro de la célula. Cuando interviene la endocitosis, en la mayoría de los casos se forma un endosoma que dirige los sistemas de administración hacia los liososomas, vesículas que contienen enzimas hidrolíticas capaces de degradar el ADN. En estos casos es conveniente que el complejo formado entre el ADN y el sistema de administración permanezca poco tiempo en el interior del endosoma, liberándose cuanto antes al citoplasma. El mecanismo de liberación va a depender del sistema de administración empleado. Los sistemas lipídicos pueden inducir la salida del endosoma mediante fusión con la membrana endosómica o mediante desestabilización de la misma[78]. Por otro lado, algunos sistemas poliméricos, como los formados con PEI y con algunos dendrímeros, presentan capacidad endosomolítica intrínseca. Estos polímeros aumentan la concentración de iones cloruro en el interior del endosoma, lo que conduce al progresivo hinchamiento del mismo, favoreciendo su ruptura[86].

Para facilitar la salida tanto de los sistemas lipídicos como de los poliméricos desde las vesículas endosómicas, se pueden incluir en las formulaciones distintas sustancias

86 Sonawane ND, Szoka FC, Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J Biol Chem. 2003; 278: 44826-44831.

que favorecen este proceso, como el DOPE[87], la cloroquina[88] y los péptidos GALA y KALA[89].

Cuando la endocitosis es mediada por caveolas, se evade la ruta intracelular que dirige los sistemas de administración hacia los lisosomas[90,91].

La entrada por una u otra vía va a estar condicionada por la célula diana, ya que algunas células pueden no presentar ciertas rutas de internalización[92], pero también por las características del sistema de administración. Rejman y col.[93] han demostrado que partículas menores de 200 nm penetran en las células B16-F10 (línea celular de melanoma de ratón) a través de clatrinas, mientras que las partículas con tamaños de 500 nm entran principalmente a través de caveolas.

3.5 ENTRADA DEL ADN EN EL NÚCLEO

Una vez que el ADN ha sido liberado al citoplasma tiene que ser capaz de llegar al núcleo. Esto no resulta fácil porque, debido a su tamaño, la movilidad es lenta. Parece ser que los endosomas pueden facilitar el acercamiento del ADN al núcleo[78]. También

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87 Farhood H, Serbina N, Huang L. The role of dioleoyl phosphatidylethanolamine in cationic liposome-mediated gene transfer. Biochim Biophys Acta. 1995; 1235: 289-295.

88 Wightman L, Kircheis R, Wagner E. Polymer-based gene delivery systems. En: Roland A, Sullivan SM, editores. Pharmaceutical gene delivery systems. New York: Marcel Dekker, Inc; 2003. p. 109-135.

89 Lee H, Jeong JH, Park TG. PEG grafted polylysine with fusogenic peptide for gene delivery: high transfection efficiency with low cytotoxicity. J Control Release. 2002; 79: 283-291.

90 Shin J-S, Abraham SN. Caveolae – not just craters in the cellular landscape. Science. 2001; 293: 1447-1448.

91 Pelkmans L, Helenius A. Endocytosis via caveolae. Traffic. 2002; 3: 311-320.

92 Fujimoto T, Kogo H, Nomura R, Une T. Isoforms of caveolin-1 and caveolar structure. J Cell Sci. 2000. 113: 3509-3517.

93 Rejman J, Oberle V, Zhuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae- mediated endocytosis. Biochem J. 2004; 377: 159-169.

puede ocurrir que el ADN se aproveche de los microtúbulos celulares para llegar al núcleo[94]. Un factor que puede disminuir la cantidad de ADN que entra en el núcleo es el ataque por parte de nucleasas citoplásmicas. El hecho de que el ADN esté incorporado en un sistema de administración hace que esté más protegido frente a la acción de esas enzimas.

Hay que tener en cuenta que para penetrar en el núcleo, el ADN primero tiene que liberarse del sistema de administración. En el caso de los sistemas lipídicos puede liberarse directamente durante el proceso de fusión con la membrana endosomal, pero en el caso de que la salida del endosoma sea por desestructuración o rotura de la membrana, la separación tendrá lugar en el citoplasma probablemente mediante la interacción con orgánulos citoplásmicos[78].

La entrada del ADN en el núcleo tampoco es sencilla, ya que la membrana nuclear supone una barrera selectiva para moléculas mayores de 40 kDa, como es el caso del ADN. Esas macromoléculas pueden entrar en el núcleo mediante dos mecanismos: durante el proceso de mitosis[95,96], cuando la membrana nuclear desaparece, o bien a través de los llamados complejos de poros nucleares (*nuclear pore complex*, NPC). En el caso de células que se dividen poco o lentamente, este segundo mecanismo es prácticamente el único a través del cual el ADN puede alcanzar el núcleo. Sin embargo, para que se produzca el transporte a través de estos poros son necesarios unos

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94 Vaughan EE, Dean DA. Intracellular trafficking of plasmids during transfection is mediated by microtubules. Mol Ther. 2006; 13: 422-428.

95 Mortimer I, Tam P, MacLachlan I, Graham RW, Saravolac EG, Joshi PB. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. Gene Ther. 1999; 6: 403-411.

96 Tseng WC, Haselton FR, Giorgio TD. Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. Biochim Biophys Acta. 1999; 1445: 53-64.

péptidos llamados señales de localización nuclear (*nuclear localization signal*, NLS). Por tanto, una posible estrategia para facilitar la entrada del ADN en el núcleo consiste en incorporar a los sistemas de administración péptidos que porten esas secuencias[31,32,33]. En el caso de los sistemas lipídicos, además, puede haber un proceso de fusión entre el sistema y la membrana nuclear que dé lugar a la liberación del ADN directamente en el núcleo[97].

97 Kamiya H, Fujimura Y, Matsuoka I, Arracima H. Visualization of intracellular trafficking of exogenous DNA delivered by cationic liposomes. Biochem Biophys Res Commun. 2002; 298: 591-597.

IV. APLICACIONES DE LA TERAPIA GÉNICA

Aunque la terapia génica se propuso inicialmente como método de tratamiento de enfermedades hereditarias causadas por la mutación de un solo gen, es decir, de enfermedades monogénicas, hoy en día se considera un método útil para el manejo de trastornos tanto hereditarios como adquiridos.

4.1 APLICACIÓN DE LA TERAPIA GÉNICA AL TRATAMIENTO DE ENFERMEDADES MONOGÉNICAS

Hasta el momento se han descrito unas 4000 enfermedades hereditarias, dentro de las cuales se encuentran un gran número de enfermedades monogénicas. En general, las enfermedades monogénicas se encuentran dentro del grupo de enfermedades raras, es decir, aquellas que afectan a un porcentaje muy pequeño de la población. Hoy en día, la búsqueda de tratamientos para este tipo de enfermedades está en auge, y la terapia génica parece ser una posibilidad interesante. La Tabla 7 recoge algunas de estas enfermedades que por su gravedad pueden ser consideradas candidatas a ser tratadas mediante terapia génica.

Tabla 7. Enfermedades hereditarias que pueden ser consideradas candidatas a ser tratadas mediante terapia génica.		
ENFERMEDAD	PRODUCTO AFECTADO	CÉLULAS DIANA
Fibrosis quística	Producto del gen CFTR	Células del pulmón
Hemofilia A	Factor de coagulación VIII	Células del hígado o fibroblastos
Hemofilia B	Factor de coagulación IX	Células del hígado o fibroblastos
Inmunodeficiencia severa combinada ligada al cromosoma X (X-SCDI)	Producto del gen IL2RG	Células madre
Inmunodeficiencia severa combinada (SCDI)	Enzima adenosin-desaminasa (ADA), RAG-1, RAG-2, JAK-3, receptor de la IL-7, CD45, CD3δ	Células de la médula ósea o linfocitos T
Retinopatías	Múltiples	Células de la retina
Enfermedad de Fabry	α-galactosidasa A lisosomal	Múltiples
Distrofia muscular de Duchenne	Distrofina	Células musculares

4.1.1 Fibrosis quística

Se trata de una enfermedad hereditaria monogénica causada por la mutación de un gen que codifica una proteína reguladora de la conductancia transmembrana: *cystic fibrosis transmembrane conductance regulator* (CFTR). Debido a este defecto, se encuentra alterado el transporte de cloruro a través de la membrana de ciertas células, afectando a glándulas secretoras del organismo. En consecuencia, las glándulas

producen una secreción mucosa espesa y pegajosa viéndose afectados diversos órganos: pulmones, páncreas, intestino, vesícula biliar y órganos reproductores.

La aplicación de la terapia génica al tratamiento de la fibrosis quística se basa en incorporar copias normales del gen que codifica el CFTR en el epitelio respiratorio, lo cual se está abordando mediante el uso tanto de vectores virales[98,99,100] como no virales[101,102,103].

4.1.2 Hemofilia

Se trata de una enfermedad congénita que afecta sólo a los hombres ya que está ligada al cromosoma X y es de carácter recesivo. Se produce como consecuencia de la falta de los factores de coagulación VIII (hemofilia A) o IX (hemofilia B), que en condiciones normales se sintetizan en el hígado. Debido a ese déficit se altera la cascada de la coagulación y los individuos que padecen esta enfermedad sufren

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98 Yonemitsu Y, Kitson C, Ferrari S, Farley R, Griesenbach U, Judd D, et al. Efficient gene transfer to airway epithelium using recombinant sendai virus. Nat Biotechnol. 2000; 18: 970-973.

99 Copreni E, Penzo M, Carrabino S, Conese M. Lentivirus-mediated gene transfer to the respiratory epithelium: a promising approach to gene therapy of cystic fibrosis. Gene Ther. 2004; 11: S67-S75.

100 Sinn PL, Burnight ER, Hickey MA, Blissard GW, McGray PB Jr. Persistent gene expression in mouse nasal epithelial following feline immunodeficiency virus-based vector gene transfer. J Virol. 2005; 79: 12818-12827.

101 Zabner J, Cheng SH, Meeker D, Launspach J, Balfour R, Perricone MA, et al. Comparison of DNA-lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia in vivo. J Clin Invest. 1997; 100: 1529-1537.

102 Sanders NN, De Smedt SC, Cheng SH, Demeester J. Pegylated GL67 lipoplexes retain their gene transfection activity after exposure to components of CF mucus. Gene Ther. 2002; 9: 363-371.

103 Konstan MW, Davis PB, Wagener JS, Hilliard KA, Stern RC, Milgram LJ, et al. Compacted DNA nanoparticles administered to the nasal mucosa of cystic fibrosis subjects are safe and demonstrate partial to complete cystic fibrosis transmembrane regulator reconstitution. Hum Gene Ther. 2004; 15: 1255-1269.

hemorragias espontáneas y prolongadas en las articulaciones, los músculos y los órganos internos.

Al igual que en el caso de la fibrosis quística, existen numerosos estudios centrados en el tratamiento mediante terapia génica de esta enfermedad con vectores virales[104,105,106] y no virales[107]. La terapia génica de la hemofilia A ha sido incluso objeto de un ensayo clínico llevado a cabo por Powell y col.[108]. Administrando por vía intravenosa a pacientes con hemofilia A severa un vector retroviral que portaba el gen del factor de coagulación humano VIII, consiguieron aumentar los niveles de este factor de coagulación y disminuir la frecuencia de aparición de hemorragias, demostrando el potencial de este tipo de tratamiento en la hemofilia A.

4.1.3 Inmunodeficiencia severa combinada (SCID)

La inmunodeficiencia severa combinada (SCID) engloba un grupo de desórdenes genéticos que cursan con un déficit de linfocitos T y B, e incluso de otras células del sistema inmunológico como las células *natural killer* (NK). Se conocen hasta diez genes

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104 Wang L, Louboutin JP, Li Y, Wilson JM. AAV7, 8, and 9 are more efficient and less immunogenic vectors for muscle-directed gene therapy for hemophilia B. Mol Ther. 2005; 11 Suppl 1: S45.

105 Ishiwata A, Mimuro J, Kashiwakura Y, Niimura M, Takano K, Ohmori T, et al. Phenotype correction of hemophilia A mice with adeno-associated virus vectors carrying the B domain-deleted canine factor VIII gene. Thromb Res. 2006; 118: 627-635.

106 Nguyen A, Dow AC, Busuttil RW, Lipshutz GS. Promoter evaluation for efficient production of human factor VIII by gene therapy in a murine model of hemophilia A. J Surg Res. 2008; 144: 439.

107 Bowman K, Sarkar R, Wang X, Mao HK, Leong KW. Evaluation of non-viral vectors for gene therapy of hemophilia A. Mol Ther. 2004; 9 Suppl 1: S314.

108 Powell JS, Ragni MV, White GC, Lusher JM, Hillman-Wiseman C, Moon TE, et al. Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. Blood. 2003; 102: 2038-2045.

responsables de esta enfermedad, diferenciándose SCID ligadas al cromosoma X (X-SCID) o SCID autosómicas recesivas. En pacientes severos este grupo de enfermedades conduce a la ausencia de respuesta inmunológica, por lo que los individuos que la padecen se encuentran expuestos a cualquier infección con posibilidad de muerte. Estas enfermedades son las que causan el síndrome de los denominados "niños burbuja".

El tratamiento común de cualquier SCID es el trasplante de médula ósea; sin embargo, las dificultades para encontrar un donante compatible han llevado a considerar la terapia génica como tratamiento alternativo.

SCID LIGADAS AL CROMOSOMA X (X-SCID): en el caso de las X-SCID la causa es el déficit del gen IL2RG. Supone casi el 50% de las SCID. Los pacientes con ese déficit no pueden producir linfocitos T, y sus linfocitos B no son capaces de sintetizar anticuerpos esenciales para combatir las infecciones. Cavazzana-Calvo y col.[109] y Gaspar y col.[110] han demostrado mediante dos ensayos clínicos que la transfección de células madre ($CD34^+$) con vectores retrovirales portando ese gen y su posterior implantación en los pacientes permite normalizar los niveles de linfocitos T. Sin embargo, dos de los pacientes incluidos en el estudio de Cavazzana presentaban una importante linfoproliferación de linfocitos T malignos[111]. Al parecer, ese efecto secundario se debe a

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109 Cavazzana-Calvo M, Hacein-Bey S, Saint Basile G, Gross F, Yvon E, Nusbaum P, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science. 2000; 288: 669-672.

110 Gaspar B, Parsley KL, Howe S, King D, Gilmour KC, Sinclair J, et al. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. Lancet. 2004; 364: 2181-2187.

111 Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science. 2003; 302: 415-419.

la inducción de mutagénesis por parte del retrovirus, a causa de la presencia del proto-oncogen LMO2. Una posible estrategia para evitar esa linfoproliferación es la incorporación de genes suicidas en el retrovirus, es decir, incorporar genes que codifiquen enzimas capaces de transformar profármacos en sustancias activas que eliminen los linfocitos que presentan esa mutación. Uno de los sistemas de genes suicidas más empleados, que ya ha sido probado en ensayos clínicos[112] es el sistema de la timidina quinasa del virus *herpes simplex*/ganciclovir (HSV-tk/GCV)[113].

SCID AUTOSÓMICAS RECESIVAS: Este grupo de inmunodeficiencias pueden ser consecuencia de la alteración de diversos genes (ADA, RAG-1, RAG-2, JAK-3, receptor de la IL-7, CD45, CD3δ) que dan lugar a problemas en la profileración de los linfocitos T y otras células del sistema inmune.

Una de ellas es la SCID debida al déficit de la enzima adenosina desaminasa (ADA), en la que el defecto de un gen hace que esa enzima no sea funcional. Como consecuencia de la falta de actividad de la enzima, se acumulan desoxiATP (dATP) y otros metabolitos asociados, dando lugar a una reducción de la actividad de la enzima ribonucleótido reductasa durante la diferenciación tímica. Disminuye la producción de linfocitos T y la supervivencia de los que se producen es menor de lo normal. El tratamiento específico clásico de esta enfermedad consiste en administrar la enzima ADA pegilada. Sin embargo, no resulta eficaz en todos los pacientes, y esto ha hecho

112 Rainov RG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. Hum Gene Ther. 2000; 11: 2389-2401.

113 Uchiyama T, Kumaki S, Ishikawa Y, Onodera M, Sato M, Du W, et al. Application of HSVtk suicide gene to X-SCID gene therapy: Ganciclovir treatment offsets gene corrected X-SCID B cells. Biochem Biophys Res Commun. 2006; 341: 391-398.

que se manejen otras alternativas, como la terapia génica. Esta fue la primera SCID tratada mediante terapia génica, y desde 1990 se han desarrollado varios ensayos clínicos. Dos de ellos consistieron en la administración a los pacientes de linfocitos T transfectados "*in vitro*"[114,115], con los que no se obtuvieron buenos resultados, ya que los pacientes siguieron necesitando el tratamiento con ADA pegilada. En otros dos ensayos se recurrió a la implantación en los pacientes de células madre hemotopoyéticas también transfectadas "*in vitro*"[116,117], y tampoco se pudo eliminar el tratamiento con la enzima. El protocolo con el que se consiguieron los mejores resultados fue aquel en el que se combinaron la administración a los pacientes de células madre hematopoyéticas transfectadas "*in vitro*" y una suave mielosupresión con busulfán, de manera que se generó espacio en la médula ósea para la generación de nuevas células con el gen del ADA[118]. Con este protocolo los pacientes han sobrevivido sin necesidad de la enzima ADA pegilada observándose que este tratamiento es seguro y eficaz a largo plazo[119].

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114 Bordingnon C, Notarangelo LD, Nobili N, Ferrari G, Casirati G, Panina P, et al. Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. Science. 1995; 270: 470-475.

115 Muul LM, Tuschong LM, Soenen SL, Jagadeesh GJ, Ramsey WJ, Long Z, et al. Persistence and expression of the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: long-term results of the first clinical gene therapy trial. Blood. 2003; 101: 2563-2569.

116 Hoogerbrugge PM, van Beusechem VW, Fischer A, Debree M, le Deist F, Perignon JL, et al. Bone marrow gene transfer in three patients with adenosine deaminase deficiency. Gene Ther. 1996; 3: 179-183.

117 Kohn DB, Hershfield MS, Carbonaro D, Shigeoka A, Brooks J, Smogorzewska EM, et al. T lymphocytes with a normal ADA gene accumulate after transplantation of transduce autologous umbilical cord blood CD34+ cells in ADA-deficient SCID neonates. Nat Med. 1998; 4: 775-780.

118 Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science. 2002; 296: 2410-2413.

119 Aiuti A, Bordignon C, Roncarolo MG. Hematopoietic stem cell gene therapy for ADA-SCID. Blood Cells, Molecules, and Diseases. 2008; 40: 248-250.

El resto de SCID autosómicas recesivas han sido menos estudiadas y hasta el momento no se han desarrollado protocolos clínicos, aunque diversos grupos han llevado a cabo estudios en ratones consiguiendo corregir inmunodeficiencias debidas, por ejemplo, a los genes JAK-3[120], RAG-2[121] y RAG-1[122].

4.1.4 Retinopatías

Dentro de las enfermedades raras de origen monogénico también se encuentran las retinopatías o enfermedades hereditarias de la retina. Los genes responsables están distribuidos por todo el genoma causando diferentes tipos de enfermedades. Algunas de ellas ya están siendo objeto de estudios con terapia génica.

La amaurosis congénita de Leber es una de la retinopatías en la que la terapia génica está experimentando mayores avances. Esta enfermedad es causada por mutaciones en el gen RPE65 que codifica una proteína de 65 KDa específica del epitelio pigmentario de la retina. Las mutaciones en este gen dan a lugar a una degeneración progresiva de la retina. Hasta el momento se ha demostrado la eficacia del tratamiento con virus adeno-asociados en ratones y perros portadores de mutaciones en el gen RPE65[123,124,125].

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120 Bunting KD, Sangster MY, Ihle JN, Sorrentino BP. Restoration of lymphocyte function in Janus Kinase 3-deficient mice by retroviral-mediated gene transfer. Nat Med. 1998; 4: 58-64.

121 Yates F, Malassis-Séris M, Stockholm D, Bouneaud C, Larousserie F, Noguiez-Hellin P, et al. Gene therapy of RAG-2-/- mice: sustained correction of the immunodeficiency. Blood. 2002; 100: 3942-3949.

122 Lagresle-Peyrou C, Yates F, Malassis-Séris M, Hue C, Morillon E, Garrigue A, et al. Long-term immune reconstitution in RAG-1-deficient mice treated by retroviral gene therapy: a balance between efficiency and toxicity. Blood. 2006; 107: 63-72.

123 Acland GM, Aguirre GD, Ray J, Zhang Q, Aleman TS, Cideciyan AV, et al. Gene therapy restores vision in a canine model of childhood blindness. Nat Genet. 2001; 28: 92-95.

Recientemente, se han administrado estos vectores a pacientes jóvenes con distrofia de retina severa debida a mutaciones en el gen RPE65[126]. Los resultados de ese estudio sugieren que la administración subretiniana de vectores adeno-asociados puede mejorar la función visual en humanos sin provocar efectos adversos inmediatos. El siguiente paso en la aplicación de la terapia génica en la amaurosis congénita de Leber es la realización de ensayos clínicos en niños con mutaciones en el gen RPE65, puesto que éstos se pueden beneficiar más que los adultos de este tipo de tratamientos.

Otro ejemplo de retinopatía que está siendo estudiada como posible objetivo de la terapia génica es la retinosquisis juvenil ligada al sexo. El gen responsable, RS1, se ha identificado en el brazo corto del cromosoma X. En este caso, la consecuencia de las mutaciones en el gen es la formación de espacios quísticos que pueden dar lugar a un desplazamiento de la capa celular de la retina, como se ve en la Figura 6.

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124 Pang J-J, Chang B, Kumar A, Nusinowitz S, Noorwez SM, Li J, et al. Gene therapy restores vision-dependent behavior as well as retinal structure and function in a mouse model of RPE65 Leber congenital amaurosis. Mol Ther. 2006; 13: 565-572.

125 Bennicelli E, Wright JF, Komaromy A, Jacobs JB, Hauck B, Zelenaia O, et al. Reversal of blindness in animal models of Leber congenital amaurosis using optimized AAV2-mediated gene transfer. Mol Ther. 2008; 16: 458-465.

126 Bainbridge JWB, Smith AJ, Barker SS, Robbie S, Henderson R, Balaggan et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. N Engl J Med. 2008; 358: 2231-2239.

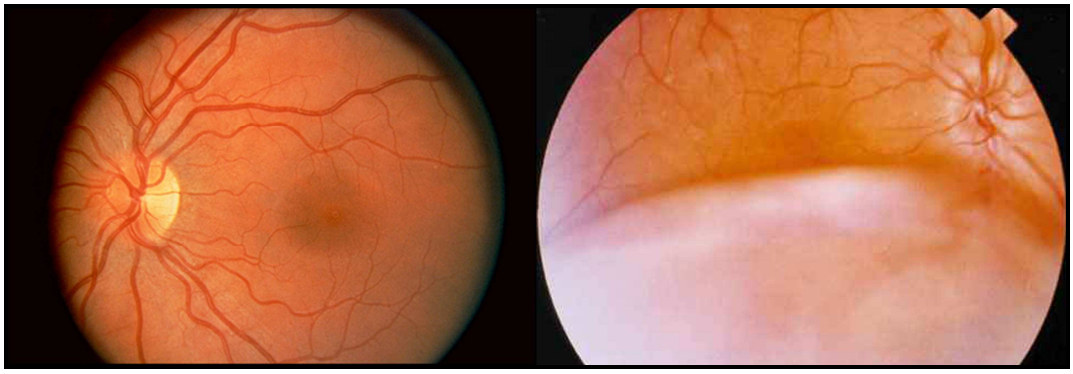

Figura 6. Fondo de ojo sano (izquierda) y con retinosquisis (derecha). En la imagen de la derecha se puede apreciar el desplazamiento de la capa celular a nivel de la retina, debido a la formación de espacios quísticos.

Dos trabajos llevados a cabo mediante la administración de virus adeno-asociados portadores de una copia del gen RS1 en animales han mostrado la recuperación de la función y la estructura de la retina, en comparación con los ojos no sometidos al tratamiento[127, 128], aunque por el momento no se ha llevado a cabo ningún estudio en humanos.

La terapia génica también está arrojando resultados prometedores en otras retinopatías como el albinismo[129], la coroideremia[130] y más recientemente en la enfermedad de Stargardt[131, 132].

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127 Min SH, Molday LL, Seeliger MW, Dinculescu A, Timmers AM, Janssen A, et al. Prolonged recovery of retinal structure/function after gene therapy in an Rs1h-deficient mouse model of X-linked juvenile retinoschisis. Mol Ther. 2005; 12: 644-651.

128 Kjellstrom S, Bush RA, Zeng Y, Takada Y, Sieving PA. Retinoschisin gene therapy and natural history in the Rs1h-KO mouse: long-term rescue from retinal degeneration. Invest Ophthalmol Vis Sci. 2007; 48: 3837-3845

129 Surace EM, Domenici L, Cortese K, Cotugno G, Di Vicino U, Venturi C, et al. Amelioration of both functional and morphological abnormalities in the retina of a mouse model of ocular albinism following AAV-mediated gene transfer. Mol Ther. 2005; 12: 652-658.

4.1.5 Enfermedad de Fabry

La enfermedad de Fabry se debe a una deficiencia congénita ligada al cromosoma X de la enzima hidrolasa lisosomal α-Galactosidasa A debido a mutaciones en el gen α-Gal A. Este defecto enzimático provoca la acumulación celular en múltiples tejidos, pero especialmente en células endoteliales, y en plasma, de glucoesfingolípidos neutros, particularmente de globotriosilceramida (Gb3), dando lugar a una sintomatología clínica muy variada y multisistémica, con afectación cardiaca, renal y neurológica que acaba comportando la muerte del enfermo a una edad temprana.

La terapia usada hasta ahora consiste en el reemplazamiento de la enzima, para lo que existen preparados comerciales a base de agalsidasa-α (Replagal®) o agalsidasa-β (Fabrazyme®) que se administran una vez cada dos semanas. Sin embargo este tratamiento es muy caro[133], por lo que es interesante buscar alternativas terapéuticas. En este sentido, la administración de genes que puedan corregir esos defectos mediante el suministro mantenido de la enzima puede ser efectiva. Yoshimitsu y col.[134] han demostrado que la transducción de células hematopoyéticas con lentivirus portadores de

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130 Anand V, Barral DC, Zeng Y, Brunsmann F, Maguire AM, Seabra MC, et al. Gene therapy for choroideremia: in vitro rescue mediated by recombinant adenosvirus. Vision Res. 2003; 43: 919-926.

131 Kong J, Kim SR, Binley K, Pata I, Doi K, Mannik J, et al. Correction of the disease phenotype in the mouse model of Stargardt disease by lentiviral gene therapy. Gene Ther. 2008; 15: 1311-1320.

132 Alloca M, Doria M, Petrillo M, Colella P, Garcia-Hoyos M, Gibbs D, et al. Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. J Clin Invest. 2008; 118: 1955-1964.

133 Masson C, Cissé I, Simon V, Insalaco P, Audran M. Fabry disease: a review. Joint Bone Spine. 2004; 71: 381-383.

134 Yoshimitsu M, Higuchi K, Ramsubir S, Nonaka T, Rasaiah VI, Siatskas C, et al. Efficient correction of Fabry mice and patient cells mediated by lentiviral transduction of hematopoietic stem/progenitor cells. Gene Ther. 2007; 14: 256-265.

cADN que codifica la enzima α-Gal A puede resultar en la corrección mantenida de la enfermedad de Fabry.

4.1.6 Distrofia muscular de Duchenne

La distrofia muscular de Duchenne es una forma de distrofia muscular causada por una anomalía en el gen que codifica la proteína distrofina presente en los músculos. Se caracteriza por la pérdida progresiva de la función muscular que comienza en las extremidades inferiores. Comúnmente cursa también con cardiomiopatía.

Actualmente no existe un tratamiento eficaz para este síndrome, por lo que se han venido estudiando distintas estrategias por parte de diversos grupos de investigación. Los vectores adenovirales permiten obtener eficiencias de transfección altas en animales con distrofia muscular[135,136]. Sin embargo, en el caso de la distrofia muscular de Duchenne la limitación en el tamaño del ADN que los vectores virales son capaces de transportar adquiere mucha importancia, ya que el gen correspondiente a la distrofina presenta un gran tamaño. Para superar este incoveniente Harper y col.[137] han desarrollado las mini- y microdistrofinas, que son genes que codifican solo ciertas porciones de la distrofina. Estos autores administraron a ratones *mdx*, que sirven como modelo de la distrofia muscular de Duchenne, virus adenoasociados portadores de

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135 Ragot T, Vincent N, Chafey P, Vigne E, Gilgenkrantz H, Coutont D, et al. Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of *mdx* mice. Nature. 1993; 361: 647-650.

136 Howell JM, Lochmuller H, O'Hara A, Fletcher S, Kakulas BA, Massie B, et al. High-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscle of dystrophic dogs: prolongation of expression with immunosuppression. Hum Gene Ther. 1998; 9: 629-634.

137 Harper SQ, Hauser MA, DelloRusso C, Duan D, Crawford RW, Phelps SF, et al. Modular flexibility of dystrophin: Implications for gene therapy of Duchenne muscular dystrophy. Nat Med. 2002; 8: 253-261.

genes de mini- y microdistrofinas, y observaron que la distrofia muscular revertía, comprobando así la funcionalidad de estos fragmentos del gen que codifica la distrofina.

4.2 APLICACIÓN DE LA TERAPIA GÉNICA AL TRATAMIENTO DE ENFERMEDADES ADQUIRIDAS

En cuanto a las enfermedades adquiridas, el cáncer, el SIDA y las enfermedades neurodegenerativas son las que están atrayendo un mayor interés en el campo de las posibles aplicaciones de la terapia génica.

4.2.1 Cáncer

A pesar de los avances e innovaciones en oncología, el cáncer continúa siendo una de las principales causas de muerte. La extirpación es el tratamiento más común contra los tumores sólidos, generalmente en combinación con radioterapia o quimioterapia. Estas técnicas dan lugar a importantes reacciones adversas que pueden afectar a órganos muy variados. La terapia génica del cáncer no se basa en corregir un defecto genético como ocurre en las enfermedades monogénicas, sino que consiste en dotar de una nueva propiedad a las células para aprovecharlas con fines terapéuticos[138].

El tratamiento del cáncer mediante terapia génica se puede abordar mediante diferentes estrategias, como se recoge en la Figura 7, diferenciándose el tratamiento a nivel inmunológico o a nivel molecular.

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138 Ronchera-Oms CL, González JM. Terapia génica. En: Bonal J, Domínguez-Gil A, Gamundi MC, Napal V, Valverde E, editores. Farmacia hospitalaria. Madrid: SCM, Doyma; 2002. p. 919-927.

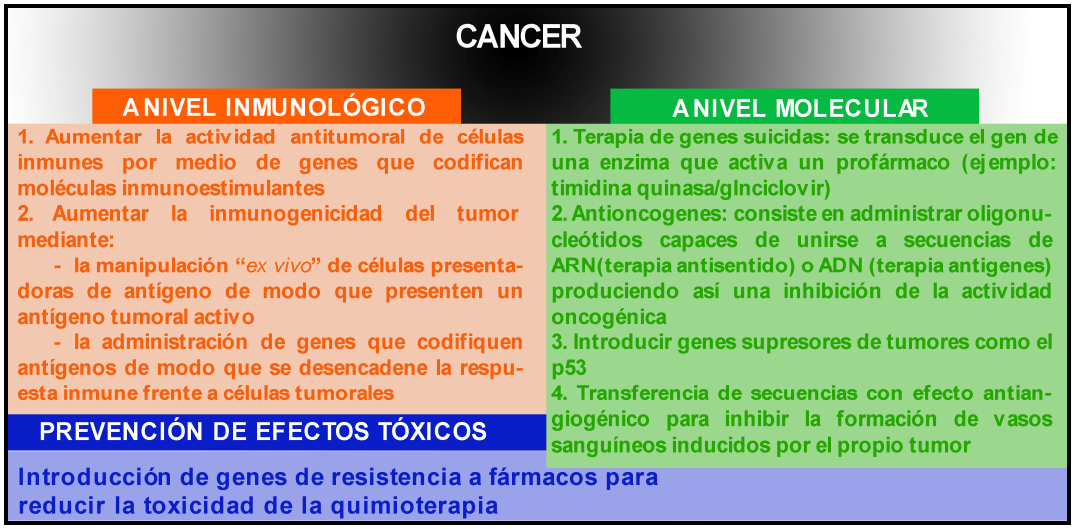

Figura 7. Resumen de las diferentes estrategias que se pueden llevar a acabo en el tratamiento del cáncer mediante terapia génica.

TRATAMIENTO A NIVEL INMUNOLÓGICO. El tratamiento desde el punto de visto inmunológico se puede conseguir de diferentes formas:

- Aumentando la actividad antitumoral de células inmunes mediante la administración de genes que codifican moléculas inmunoestimulantes como la IL12. Diversos autores han demostrado la eficacia de esta estrategia frente a tumores renales y adenocarcinomas[139,140] y frente a células de cáncer pulmonar de

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139 Shi F, Rakhmilevich AL, Heise CP, Oshikawa K, Sondel PM, Yang NS, et al. Intratumoral injection of interleukin-12 plasmid DNA, either naked or in complex with cationic lipid, results in similar tumor regression in murine model. Mol Cancer Ther. 2002; 1: 949-957.

140 Janát-Amsbury M, Yockman JW, Lee M, Kern S, Furgeson DY, Bikrama M, et al. Local, non-viral IL-12 gene therapy using a water soluble lipopolymer as carrier system combined with systemic paclitaxel for cancer treatment. J Control Release. 2005; 101: 273-285.

ratón[141].

- Aumentando la inmunogenicidad del tumor trabajando con antígenos tumorales activos.

  Se pueden manipular "*ex vivo*" células presentadoras de antígeno, de modo que sinteticen y presenten esos antígenos tumorales[142], o bien se pueden administrar genes que codifiquen los antígenos[143]. Con ambas estrategias se puede conseguir que se desencadene una respuesta inmune contra esos antígenos, y por tanto contra las células cancerígenas que los poseen de manera natural.

  Zhu y col.[144], en cambio, han recurrido al gen de la citoquina MP-3α que es capaz de atraer células dendríticas al tumor, consiguiéndose así la activación de la respuesta inmune frente al tumor.

TRATAMIENTO A NIVEL MOLECULAR. Las estrategias que se han propuesto hasta ahora son:

- Introducción de genes suicidas, es decir, genes que codifican una enzima que

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141 Jin HS, Park EK, Lee JM, NamKoong SE, Kim DG, Lee YJ, et al. Immunization with adenoviral vectors carrying recombinant IL-12 and E7 enhanced the antitumor immunity to human papillomavirus 16-associated tumor. Gynecol Oncol. 2005; 97:559-567.

142 Vollmer Jr. CM, Eilber FC, Butterfield LH, Ribas A, Dissette VB, Koh A, et al. α-fetoprotein-specific genetic immunotherapy for hepatocellular carcinoma. Cancer Res. 1999; 59: 3064-3067.

143 Tang H, Tang XY, Liu M, Li X. Targeting alpha-fetoprotein represses the proliferation of hepatoma cells via regulation of the cell cycle. Clin Chim Acta. 2008; 394: 81-88.

144 Zhu B, Zou L, Cheng X, Lin Z, Duan Y, Wu Y, et al. Administration of MIP-3α gene to the tumor following radiation therapy boosts anti-tumor immunity in a murine model of lung carcinoma. Immun Lett. 2006; 103: 101-107.

activa selectivamente un profármaco. En el tratamiento del cáncer se ha empleado el sistema de la timidina quinasa del virus *herpes simplex*/ganciclovir (HSV-tk/GCV)[145], el sistema de la citosina desaminasa (CD) que activa la fluorocitosina[146] y la combinación de ambos que permite usar a la vez como profármacos fluorocitosina y ganciclovir[147].

- Administración de antioncogenes. Se trata de oligonucleótidos capaces de unirse a secuencias de ARN (terapia antisentido) o ADN (terapia antigenes), produciendo así una inhibición de la actividad oncogénica. Los oncogenes c-erbB2 y el c-myc, por ejemplo, son frecuentes en el cáncer de ovario. Por lo tanto, una posible estrategia para el tratamiento de este tipo de cáncer será la administración de los correspondientes antioncogenes. Fei y col. estudiaron "*in vitro*" la potencia en el tratamiento de la administración conjunta de esos antioncogenes[148], observando que las células de cáncer de ovario humano en cultivo crecen más lentamente cuando son tratadas con ambos antioncogenes, en comparación con el tratamiento con cada uno de ellos por separado o la ausencia de tratamiento.
- Introducción de genes que codifican factores supresores de tumores. Uno de los

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145 Gerolami R, Uch R, Faivre J, Garcia S, Hardwigsen J, Cardoso J, et al. Herpes simplex virus thymidine kinase-mediated suicide gene therapy for hepatocellular carcinoma using HIV-1-derived lentiviral vectors. J Hepatol. 2004; 40: 291-297.

146 Chaszczewska-Markowska M, Stebelska K, Sikorski A, Madej J, Opolski A, Ugorski M. Liposomal formulation of 5-fluorocytosine in suicide gene therapy with cytosine deaminase – for colorectal cancer. Cancer Lett. 2008; 262: 164-172.

147 Freytag SO, Paielli D, Wing M, Rogulski K, Brown S, Kolozsvary A, et al. Efficacy and toxicity of replication-competent adenovirus-mediated double suicide gene therapy in combination with radiation therapy in an orthotopic mouse prostate cancer model. Int J Rad Oncol Biol Phys. 2002; 54: 873-885.

148 Fei R, Shaoyang L. Combination antigene therapy targeting c-myc and c-erbB2 in the ovarian cancer COC1 cell line. Gynecol Oncol. 2002; 85: 40-44.

más representativos es el de la proteína p53. Existen estudios en los que se han obtenido buenos resultados combinando la administración del gen de la proteína p53, junto con radioterapia[149] o con quimioterapia[150].

- Introducción de factores con efecto antiangiogénico, es decir, factores que inhiben el desarrollo de nuevos vasos que irriguan los tumores, ya que los tumores requieren aporte sanguíneo para crecer y llegar a alcanzar un tamaño crítico. Existen moléculas que tienen actividad antiangiogénica conocida, y diversos autores han comprobado que la terapia génica destinada a la producción de estas moléculas, como la endostatina[151,152], la angioestatina[151,152], el NK4[152] o la vasostaina[153] permiten reducir el crecimiento de diversos tumores.

  Sin embargo, Cui y col.[154] han detectado el crecimiento de un tumor tratado con endostatina, al parecer debido a que esta molécula también tiene capacidad de

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149 Swisher SG, Roth JA, Komaki R, Gu J, Lee JJ, Hicks M, et al. Induction of p53-regulate genes and tumor regression in lung cancer patients after intratumoral delivery of adenoviral p53 (INGN 201) and radiation therapy. Clin Cancer Res. 2003; 9: 93-101.

150 Nemunaitis J, Swisher SG, Timmons T, Connors D, Mack M, Doerksen L, et al. Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. J Clin Oncol. 2000; 18: 609-622.

151 Schmitz V, Wang L, Barajas M, Gomar C, Prieto J, Qian C. Treatment of colorectal and hepatocellular carcinomas by adenoviral mediated gene transfer of endostatin and angiostatin-like molecule in mice. Gut. 2004; 53: 561-567.

152 Matsumoto G, Kubota E, Tabata Y. Antiangiogenic gene therapy combined with low-dose chemotherapy suppresses murine squamous cell carcinoma. Oral Oncol. 2007; 2-S1: 186.

153 Li L, Yuan Y-Z, Lu J, Xia L, Zhu Y, Zhang Y-P, et al. Treatment of pancreatic carcinoma by adenoviral mediated gene transfer of vasostatin in mice. Gut. 2006; 55: 259-265.

154 Cui R, Takahashi K, Takahashi F, Tanabe KK, Fukuchi Y. Endostatin gene transfer in murine lung carcinoma cells induces vascular endothelial growth factor secretion resulting in up-regulation of in vivo tumorigenecity. Cancer Lett. 2006; 232: 262-271.

inducir la secreción del factor de crecimiento del endotelio vascular (VEGF), y por tanto favorece la neovascularización. Por otro lado, Matsumoto y col.[152] han conseguido resultados esperanzadores en ratones tratados con una combinación de los genes que codifican la endostatina, angiotensina y NK4 y dosis bajas de quimioterapia.

Al margen de estas estrategias inmunológicas o moleculares también puede resultar útil en el tratamiento del cáncer la introducción de genes de resistencia a fármacos para reducir la toxicidad debida a los fármacos antineoplásicos, como la mielosupresión. Uno de esos genes es el MDR-1, que codifica un transportador de la membrana celular, la glicoproteína P, que expulsa moléculas hidrófobas y anfipáticas. Estudios preclínicos en los que se han transfectado células sanguíneas con ese gen confirman la viabilidad de esta estrategia[155].

4.2.2 SIDA

El síndrome de inmunodeficiencia adquirida o SIDA, producido por el virus VIH, ha matado y sigue matando millones de personas. A pesar del desarrollo de los fármacos antirretrovirales, la posible mutación del virus y la generación de cepas resistentes a estos fármacos, hace necesario el desarrollo de nuevas terapias. La terapia génica puede tener un papel importante en la lucha contra el sida.

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155 Schiedlmeier B, Schilz AJ, Kühlcke K, Laufs S, Baum C, Zeller WJ, et al. Multidrug resistance 1 gene transfer can confer chemoprotection to human peripheral blood progenitor cells engrafted in immunodeficient mice. Hum Gene Ther. 2002; 13: 233-242.

El ciclo de vida del VIH, representado en la Figura 8, tiene múltiples etapas, algunas de las cuales son susceptibles de ser interrumpidas mediante la administración de genes.

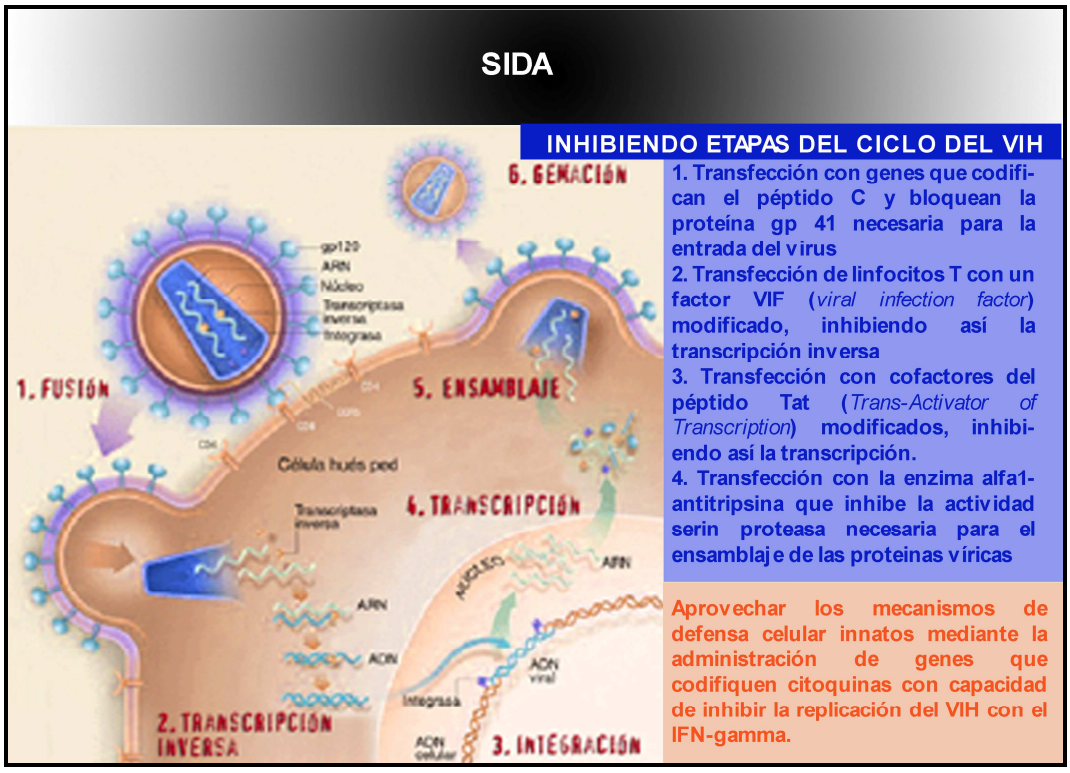

Figura 8. Izquierda: esquema del ciclo de vida del VIH. Derecha: resumen de las estrategias de tratamiento del SIDA mediante terapia génica.

- Entrada celular. El VIH presenta una glicoproteína transmembrana llamada gp41 que media la fusión del virus con la membrana celular para entrar en la célula. Egelhofer y col.[156] han llevado a cabo una modificación genética de células que

156 Egelhofer M, Brandenburg G, Martinius H, Schult-Dietrich P, Melikyan G, Kunert R, et al. Inhibition of human immunodeficiency virus type 1 entry in cells expressing gp41-derived peptides. J Virol. 2004; 78:568-575.

son infectadas por el VIH, de manera que expresan péptidos C que se unen a la proteína gp41 y bloquean la entrada del virus en la célula.

- Transcripción inversa. Otra proteína característica de los lentivirus es la conocida como Vif (*Viral Infectivity Factor*). Este factor es necesario para que el virus sea infectivo, ya que al parecer bloquea los sistemas antivirales innatos de las células inmunes. Stopak y col.[157] han comprobado que el mecanismo de acción de este factor es el bloqueo de la enzima APOBEC3G. Esta enzima presenta actividad citidina desaminasa, y se encarga de modificar las moléculas de ADN que resultan de la transcripción inversa. Esa modificación es la responsable de la mutación vírica que inhibe su capacidad infectiva. Vallanti y col.[158] han propuesto la transfección de linfocitos T con un factor Vif modificado, llamado F12-Vif, con el que han conseguido bloquear la infectividad del VIH en estas células, pero al parecer a través de un mecanismo diferente al de la APOBEC3G.

- Transcripción. El factor Tat (*Trans-Activator of Transcription*) es también muy importante en el ciclo de vida del VIH, ya que estimula la transcripción, y por tanto es un regulador positivo de la síntesis de proteínas víricas. Este trans-activador requiere la actuación de cofactores como la ciclina T o el CDK9. Fujinaga y col.[159]

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157 Stopak K, de Noronha C, Yonemoto W, Green WC. HIV-1 vif blocks the antiviral activity of APOBEC3G by imparing both its translation and intracellular stability. Mol Cell. 2003; 12: 1-20.

158 Vallanti G, Lupo R, Federico M, Mavilio F, Bovolenta C. T lymphocytes transduced with a lentiviral vector expressing F12-vif are protected from HIV-1 infection in an APOBEC3G-independent manner. Mol Ther. 2005; 12: 697-706.

159 Fujinaga K, Irwin D, Geyer M, Peterlin M. Optimized chimeras between kinase-inactive mutant Cdk9 and truncated cyclin T1 proteins efficiently inhibit Tat transactivation and human immunodeficiency virus gene expression. J Virol. 2002; 76: 10873-10881.

han demostrado que la cotransfección con formas mutadas de estos cofactores inhibe la función del Tat y la expresión génica del VIH.

- Procesamiento de las proteínas virales. El HIV necesita el procesamiento de la proteína gp160 que es precursora de las proteínas gp120 y gp41. Éstas son proteínas de membrana responsables de la unión del virus a la superficie celular a través del receptor CD4. Para que se lleve a cabo ese procesamiento es necesaria actividad serin proteasa. Cordelier y col.[160] han visto "*in vitro*" que la expresión de la α1-antitripsina (α1AT), que tiene actividad antiserin proteasa, puede resultar eficaz para inhibir la replicación del VIH.

  En todo este proceso hay que tener en cuenta otro aspecto. El receptor CD4 empleado habitualmente por el VIH para unirse a la superficie celular, también parece inhibir la infectividad de las partículas víricas liberadas[161], por lo que el virus ha desarrollado un mecanismo para retirar esta molécula de la superficie de las células infectadas. Pham y col.[162] han podido comprobar que la infectividad se reduce notablemente en células a las que se transfecta con moléculas CD4 resistentes a ese mecanismo desarrollado por el virus.

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160 Cordelier P, Strayer DS. Conditional expression of α1-antitrypsin delivered by recombinant SV40 vectors protects lymphocytes against HIV. Gene Ther. 2003; 10: 2153-2156.

161 Lama J, Mangasarian A, Trono D. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- an Vpu-inhibitable manner. Curr Biol. 1999; 9: 622-631.

162 Pham HM, Argañaraz ER, Groschel B, Trono D, Lama J. Lentiviral vectors interfering with virus-induced CD4 down-modulation potently block human immunodeficiency virus type 1 replication in primary lymphocytes. J Virol. 2004; 78: 13072-13081.

Otra posibilidad es aprovechar los mecanismos de defensa celular innatos, por ejemplo mediante la inducción de expresión génica de citoquinas como el IFN-α2 que tiene capacidad de inhibir la replicación del VIH[163,164,165].

4.2.3 Enfermedades neurodegenerativas

La terapia génica también es aplicable al tratamiento de enfermedades neurodegenerativas como el Alzheimer o el Parkinson (Figura 9), cada vez más frecuentes en nuestra sociedad y que junto con las enfermedades circulatorias y tumores, constituyen la causa de muerte más importante en la población española[166].

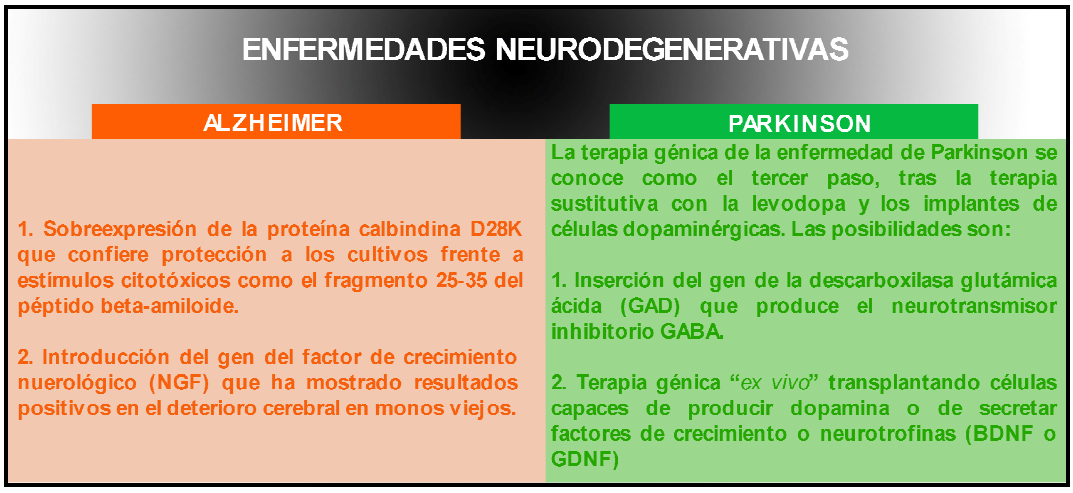

Figura 9. Resumen de las estrategias de tratamiento mediante terapia génica del Alzheimer y el Parkinson.

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163 Cordelier P, Calarota SA, Strayer DS. Trans-activated interferon-α2 delivered to T cells by SV40 inhibits early stages in the HIV-1 replicative cycle. J Hematother Stem Cell Res. 2002; 11: 817-828.

164 Cordelier P, Calarota SA, Pomerantz RJ, Xiaoshan J, Strayer DS. Inhibition of HIV-1 in the central nervous system by IFN-α2 delivered by an SV40 vector. J Interferon Cytokine Res. 2003; 23: 477-488.

165 Brule F, Khatissina E, Benani A, Bodeux A, Montagnier L, Piette J, et al. Inhibition of HIV replication: A powerful antiviral strategy by IFN-β gene delivery in CD4+ cells. Biochem Pharmacol. 2007; 74: 898-910.

166 Jordán J. Avances en el tratamiento de las enfermedades neurodegenerativas. Offarm. 2003; 3: 102-112.

ALZHEIMER. La terapia génica se vislumbra como un tratamiento prometedor para frenar el deterioro cognitivo que padecen los enfermos de Alzheimer.

Una de las líneas de trabajo utiliza la sobreexpresión de la proteína calbindina D28K para proteger las neuronas de estímulos citotóxicos como el fragmento 25-35 del péptido β-amiloide, consiguiéndose altos rendimientos "*in vitro*"[167].

Otra posibilidad consiste en inyectar directamente en el cerebro células epiteliales modificadas genéticamente para que secreten el factor de crecimiento nervioso (NGF)[168], una proteína que previene la muerte celular. Se ha llevado a cabo un ensayo clínico en fase I[169] siguiendo esta estrategia demostrándose mejoría en 6 de los pacientes estudiados. Sin embargo, estos resultados preliminares requieren una mayor investigación, sin olvidar los riesgos que la técnica de administración puede conllevar.

PARKINSON. La terapia génica de esta enfermedad ha recibido el nombre del "tercer paso", después de la terapia sustitutiva con levodopa y los implantes de células dopaminérgicas.

Kapplit y col. han llevado a cabo un ensayo clínico en fase I administrando en el núcleo subtalámico virus adenoasociados con el gen que codifica la enzima

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167 Monje ML, Phillips R, Sapolsky R. Calbindin overexpression buffers hippocampal cultures from the energetic impairments caused by glutamate. Brain Res. 2001; 911: 37-42.

168 Tuszynski MH, Roberts J, Senut MC, U HS, Gage FH. Gene therapy in the adult primate brain: intraparenchymal grafts of cells genetically modified to produce nerve growth factor prevent cholinergic neuronal degeneration. Gene Ther. 1996; 3:305.

169 Tuszynski MH, Thal L, Pay M, Salmon DP, U HS, Bakay R, et al. A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat Med. 2005; 11:551.

descarboxilasa glutámica ácida (GAD)[170]. Esta enzima es responsable de la producción del neurotransmisor inhibitorio GABA, y su producción a nivel de las células hiperactivas del núcleo subtalámico resulta en un funcionamiento más normal de la actividad cerebral.

También se están realizando trabajos de terapia génica "*ex vivo*" en los que se administran células capaces de producir dopamina[171] o secretar factores de crecimiento como el BDNF[172] o el GDNF[173], que activan rutas de supervivencia celular en las neuronas.

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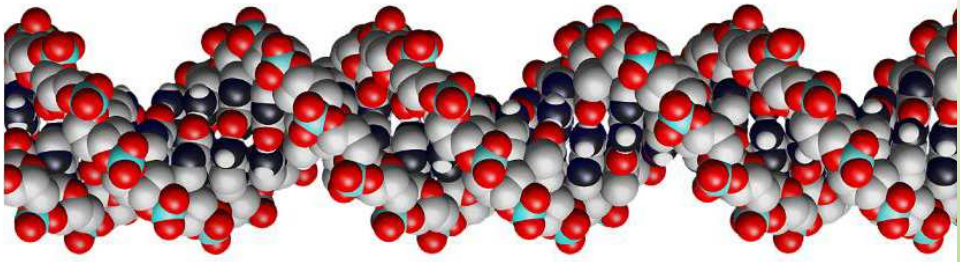
170 Kaplitt MG, Feigin A, Tang C, Fitzsimons HL, Mattis P, Lawlor PA, et al. Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne *GAD* gene for Parkinson's disease: an open label, phase I trial. Lancet 2007; 369: 2097–2105.

171 Cho YH, Kim D-S, Kim PG, Hwang YS, Cho MS, Moon SY, et al. Dopamine neurons derived from embryonic stem cells efficiently induce behavioral recovery in a Parkinsonian rat model. Biochem Biophys Res Commun. 2006; 341: 6-12.

172 Frim DM, Uhler TA, Galpern WR, Beal MF, Breakefield XO, Isacson O. Implanted fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevent 1-methyl-4-phenylpyridinium toxicity to dopaminergic neurons in the rat. Proc Natl Acad Sci. 1994; 91: 5104–5108.

173 Zurn AD, Widmer HR, Aebischer P. Sustained delivery of GDNF: towards a treatment for Parkinson's disease. Brain Res Rev. 2001; 36: 222-229.

OBJETIVOS



La terapia génica se perfila como una potente herramienta terapéutica para la cura de enfermedades para las que actualmente sólo existe tratamiento sintomático

Los vectores no virales, entre los que se incluyen las nanopartículas sólidas lipídicas (SLNs), permiten la administración de material genético tanto de forma local como sistémica. Además, presentan importantes ventajas frente a los vectores virales ya que presentan un menor riesgo de inmunogenicidad y capacidad oncogénica. El gran reto actual de la terapia génica con vectores no virales es mejorar la baja eficiencia de transfección, por lo que son necesarios grandes esfuerzos en la optimización y desarrollo de nuevos vectores no virales seguros y eficaces.

OBJETIVOS

El objetivo principal de la presente Tesis Doctoral es el desarrollo de nanopartículas sólidas lipídicas (SLNs) como sistemas de transfección no viral. La consecución de este objetivo se llevará a cabo en diferentes etapas:

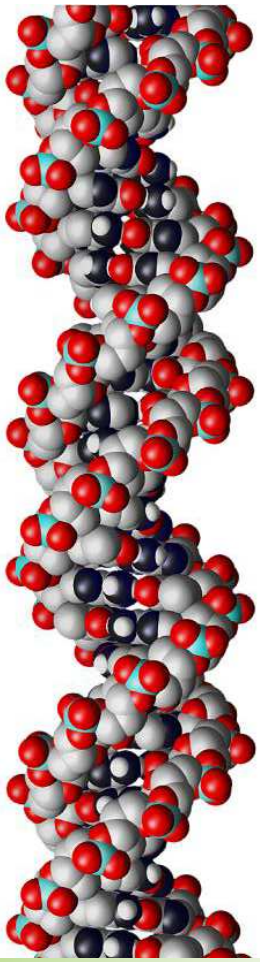
1. Optimización de la eficacia de transfección mediante el estudio de los factores relacionados con la formulación que afectan a la capacidad de transfección "*in vitro".*

2. Caracterización de los procesos de internalización y distribución intracelular de los vectores.

3. Estudio de la influencia de la incorporación de un péptido de penetración celular en la eficacia de transfección "*in vitro*".

4. Liofilización y estudio de estabilidad de los vectores a corto y largo plazo en condiciones controladas de humedad y temperatura.

5. Evaluación de la capacidad de transfección "*in vivo*" de los vectores tras su administración a ratones por vía endovenosa.



DESARROLLO EXPERIMENTAL

SOLID LIPID NANOPARTICLES: FORMULATION FACTORS AFFECTING CELL TRANSFECTION CAPACITY

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ABSTRACT

Since solid lipid nanoparticles (SLNs) were introduced as non-viral transfection systems, very few reports of their use for gene delivery have been published. In this work different formulations based on SLN–DNA complexes were formulated in order to evaluate the influence of the formulation components on the "*in vitro*" transfection capacity. SLNs composed by the solid lipid Precirol® ATO 5, the cationic lipid DOTAP and the surfactant Tween-80, and SLN–DNA complexes prepared at different DOTAP:DNA ratios were characterized by studying their size, surface charge, DNA protection capacity, transfection and cell viability in HEK293 cultured cells. The incorporation of Tween-80 allowed for the reduction of the cationic lipid concentration. The formulations prepared at DOTAP:DNA ratios 7:1, 5:1 and 4:1 provided almost the same transfection levels (around 15% transfected cells), without significant differences between them ($p > 0.05$). Other assayed formulations presented lower transfection. Transfection activity was dependent on the DOTAP:DNA ratio since it influences the DNA condensation into the SLNs. DNA condensation is a crucial factor which conditions the transfection capacity of SLNs, because it influences DNA delivery from nanoparticles, gene protection from external agents and DNA topology.

Keywords: Solid lipid nanoparticles; Non-viral vectors; Gene therapy; "*In vitro*" transfection

1. INTRODUCTION

Gene therapy is a rapidly advancing field with great potential for the treatment of genetic and acquired systemic diseases. This therapy requires the introduction of foreign DNA into the target cells and a gene delivery system must be used to facilitate the cellular uptake and the intracellular processing of the exogenous DNA. Gene delivery systems include viral vectors and non-viral vectors. Viral vectors are the most effective, but their application is limited by their immunogenicity, oncogenicity and the small size of the DNA they can transport. Non-viral vectors, however, are safer, lowered cost, and more reproducible and do not present DNA size limit. The main problem of non-viral systems is their low transfection efficiency.

Non-viral transfection systems may be composed by cationic peptides, cationic polymers or cationic lipids, and the combination of some of those components is also possible[1,2].

Although there is a large number of publications about cationic liposomes and cationic lipid emulsions for gene therapy, only a few reports about the use of solid lipid nanoparticles (SLNs) for delivery of genes[3,4,5,6] have been published since Olbrich et al.[7]

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1 Tokunaga M, Hazemoto N, Yotsuyanagi T. Effect of oligopeptides on gene expression: comparison of DNA/peptide and DNA/peptide/liposome complexes. Int J Pharm. 2004; 269: 71-80.

2 Hyndman L, Lemoine JL, Huang L, Porteous DJ, Boyd AC, Nan XS. HIV-1 Tat protein transduction domain peptide facilitates gene transfer in combination with cationic liposomes. J Control Release. 2004; 99: 435-444.

3 Tabatt K, Kneuer C, Sameti M, Olbrich C, Muller RH, Lehr CM, et al. Transfection with different colloidal systems: comparison of solid lipid nanoparticles and liposomes. J Control Release. 2004; 97: 321-332.

4 Tabatt K, Sameti M, Olbrich C, Muller RH, Lehr CM. Effect of cationic lipid and matrix lipid composition on solid lipid nanoparticle-mediated gene transfer. Eur J Pharm Biopharm. 2004; 57: 155-162.

introduced these particles as a non-viral transfection system. From the point of view of application, SLNs have good stability[8], which facilitates the industrial elaboration and the manipulation for different processes such as lyophilization.

In most cases the elaboration of SLNs in addition to the matrix lipid and the cationic lipid requires additional surfactants. Tween 80 is one of the most employed surfactants in pharmaceutical industry, and it has some interesting characteristics to be used in formulations for gene therapy, because of the presence of poly(ethyleneglycol) (PEG) chains in its structure. Different research groups have observed that the presence of PEG in cationic lipid emulsions[9,10] and in liposomes[11] improves their transfection capacity. Liu et al.[9] showed that Tween-80 was the most effective non-ionic surfactant to avoid the formation of aggregates. In their opinion, when complexes are formed each molecule of DNA may bind more than one emulsion particle such that large aggregates are formed. However, Tween-80 may prevent sterically each DNA molecule from binding to more than

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5 Rudolph C, Schillinger U, Ortiz A, Tabatt K, Plank C, Müller R, et al. Application of novel solid lipid nanoparticles (SLN)-gene vector formulations based on a dimeric HIV-1 Tat-peptide in vitro and in vivo. Pharm Res. 2004; 21: 1662-1669.

6 Pedersen N, Hansen S, Heydenreich AV, Kristensen HG, Poulsen HS. Solid lipid nanoparticles can effectively bind DNA, streptavidin and biotinylated ligands. Eur J Pharm Biopharm. 2006; 62: 155-162.

7 Olbrich C, Bakowsky U, Lehr CM, Muller RH, Kneuer C. Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA. J Control Release. 2001; 77: 345-355.

8 Freitas C, Müller RH. Correlation between long-term stability of solid lipid nanoparticles (SLN™) and crystallinity of the lipid phase. Eur J Pharm Biopharm. 1999; 47: 125-132.

9 Liu F, Yang J, Huang L, Liu D. Effect of non-ionic surfactants on the formation of DNA/emulsion complexes and emulsion-mediated gene transfer. Pharm Res. 1996; 13: 1642-1646.

10 Liu F, Yang J, Huang L, Liu D. New cationic lipid formulations for gene transfer. Pharm Res. 1996; 13: 1856-1860.

11 Meyer O, Kirpotin D, Hong KL, Sternberg B, Park JW, Woodle MC, et al. Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides. J Biol Chem. 1998; 273: 15621-15627.

one particle and the formation of such large aggregates does not occur. Besides, Tween 80 has another important characteristic for the transfection of these systems "*in vivo*". It creates a steric barrier[12] which neutralizes the excess of positive charges of the systems and reduces the interaction with blood components, such as serum proteins, which could limit the arrival of the gene therapy system to the cell surface.

In order to form lipoplexes the positive superficial charge of the systems is necessary to electrostatically bind the DNA, which has negative charge. When DNA binds with these systems it is condensed, and that condensation increases as the charges ratio (+:-) increases[13]. Condensation is necessary to facilitate the mobility of DNA molecules, which is limited by their large size, and to protect the DNA from agents present inter and intracellulary. Condensation reduces the exposure of the DNA to those agents and improves its protection. However, Faneca et al.[13] also indicated that DNA condensation may limit the transfection efficiency of non-viral systems because the larger the condensation the more difficult the release of the DNA from the complexes.

The objective of this study was to evaluate the influence of the composition of SLN-DNA complexes for gene therapy on their transfection capacity of culture cells. These non-viral vectors have not been studied as extensively as liposomes for gene therapy, and the aim of this work was to evaluate some relevant formulation factors which may be important to improve their application in gene therapy. The influence of composition of SLNs and DOTAP:DNA ratio was studied.

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12 Harvie P, Wong Frances MP, Bally Marcel B. Use of poly(ethylene glycol)-lipid conjugates to regulate the surface attributes and transfection activity of lipid-DNA particles. J Pharm Sci. 2006; 89: 652-663.

13 Faneca H, Simoes S, de Lima MCP. Evaluation of lipid-based reagents to mediate intracellular gene delivery. Biochim Biophys Acta. 2002; 1567: 23-33.

2. MATERIALS AND METHODS

2.1 Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). *N*-[1-(2,3-Dioleoyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), deoxyribonuclease I (DNase I), lauryl sulfate sodium (SDS), antibiotic/antimycotic and DNA from salmon sperm were purchased from Sigma–Aldrich (Madrid, Spain). Tween-80 was provided by Vencaser (Bilbao, Spain) and dichloromethane by Panreac (Barcelona, Spain).

Plasmid pCMS-EGFP encoding the enhanced green fluorescent protein (EGFP) was purchased from BD Biosciences Clontech (Palo Alto, US) and amplified by Dro Biosystems S.L. (San Sebastián, Spain).

The materials employed for the electrophoresis on agarose gel were acquired from Bio-Rad (Madrid, Spain). PicoGreen® dsDNA quantitation reagent (PicoGreen®) was provided by Molecular Probes (Oregon, US).

The cell culture reagents were purchased from LGC Promochem (Barcelona, Spain).

BD Viaprobe kit was provided by BD Biosciences (Belgium).

2.2 SLN production

The SLNs were produced by a solvent emulsification/evaporation technique[14]. The lipid Precirol® ATO 5 was dissolved in the organic solvent dichloromethane (5%, w/v), and then emulsified in an aqueous phase that contained the cationic lipid DOTAP and the surfactant Tween-80. Different concentrations (w/v) of DOTAP and Tween-80 were

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14 Mehnert W, Mader K. Solid lipid nanoparticles - Production, characterization and applications. Adv Drug Deliv Rev. 2001; 47: 165-196.

used—formulation 1: DOTAP 0.4% and Tween-80 0.1%; formulation 2: DOTAP 0.3% and Tween 80 0.2%; formulation 3: DOTAP 0.3% and Tween-80 0.1%. The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) during 30 s at 50W. The organic solvent then was removed from the emulsion by evaporation using a magnetic agitator for 45 min followed by vacuum conditions for 15 min. Upon dichloromethane evaporation SLNs suspension was formed by precipitation of the Precirol® ATO 5 in the aqueous medium.

Finally, SLNs were washed by centrifugation (3000 rpm, 20 min, three times) using the Amicon® Ultra centrifugal filters (Millipore, Madrid, Spain).

2.3 Preparation of SLN-DNA complexes

Twenty-five microliters of pCMS-EGFP plasmid DNA solution at 2μg/μL concentration was mixed with different amounts of SLNs suspensions in Milli-Q™ water. The amounts of SLNs required for complete DNA binding were determined individually by agarose gel electrophoresis and expressed as w/w ratio of DOTAP:DNA. DOTAP:DNA ratios assayed ranged from 15:1 to 1:1. SLN–DNA complexes were prepared by mixing DNA solution and SLNs suspension during 30 min at 25 ºC.

2.4 Size and zeta potential measurements

The sizes of SLNs and SLN–DNA complexes were determined by photon correlation spectroscopy (PCS). Zeta potential was measured by laser doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in NaCl 0.1 mM.

2.5 Atomic force microscopy

SLNs and SLN–DNA complexes were observed by atomic force microscopy (AFM) using the Multimode™ model from Digital Instruments. The images were captured in Tapping Mode™ using a cantilever of silicon rotated tapping etched silicon probe type (RTESP) with a resonance frequency of about 300 kHz.

2.6 Agarose gel electrophoresis

SLN–DNA complexes were diluted in water Milli-Q™ to a final concentration of 0.1 μgDNA/μL and subjected to electrophoresis on an agarose gel (1% ethidium bromide included for visualization) for 30 min at 120V. The bands were observed with a model TFX-20M transilluminator (Vilber-Lourmat). Images were captured using a digital camera from Bio-Rad, DigiDoc model.

2.7 Quantitation of DNA binding: PicoGreen assay

SLN–DNA complexes were washed by centrifugation using the PVDF centrifugal filters Ultrafree® MC GV 0.22 μm (Millipore, Madrid, Spain). The filtered phase during the centrifugation was recovered and mixed with the PicoGreen® reagent. The fluorescence of the samples was measured by a SFM 25 fluorometer (excitation 480 nm, emission 520 nm). From the measured fluorescence we calculated the amount of DNA which was not bound to the SLNs.

2.8 DNase I protection study

DNase I was added to SLN–DNA complexes to a final concentration of 1U DNase I/2.5μg DNA, and the mixtures were incubated at 37 ºC for 30 min. Afterwards 2% SDS solution was added to the samples to a final concentration of 1% to release DNA from

SLNs. Samples were then analysed by electrophoresis on agarose gel and the integrity of the DNA in each sample was compared with untreated DNA as control.

2.9 Cell culture and transfection protocol

The Human Embryonic Kidney (HEK293) cell line was obtained from the American Type Culture Collection (ATCC) and maintained in Eagle's Minimal Essential Medium with Earle's BSS and 2mM L-glutamine (EMEM) supplemented with 10% heat-inactivated horse serum and 1% antibiotic/antimycotic. Cells were incubated at 37 ºC with 5% CO_2 in air and subcultured every 2–3 days using trypsin/EDTA.

For transfection HEK293 cells were seeded on 24-well plates at a density of 150,000 cells per well and allowed to adhere overnight. Seventy-five microliters of the complexes solution (2.5 μg DNA) was added, and cells were incubated with the complexes for 4 h at 37 ºC. The medium containing the complexes in the wells was diluted with 1mL of complete medium and cells were allowed to grow for further 72 h.

As a blank we used SLN–DNA complexes formulated in the same conditions but with DNA from salmon sperm instead of pCMS-EGFP plasmid.

2.10 Qualitative analysis of transfection efficacy: fluorescent microscopy

Detection of expression of EGFP was carried out at 72 h post-transfection using an inverted microscope equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon). Observations and image captures were performed using a 20× objective.

2.11 Quantitative analysis of transfection efficacy and cytotoxicity: flow cytometry

At the end of the incubation, cells were washed once with 300 μL of PBS and were detached with 300 μL of trypsin/EDTA. Then the cells were centrifuged at 1500*g* and the supernatant was discarded. The cells were re-suspended with PBS and directly introduced to a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, USA). For each sample 10,000 events were collected.

For transfection efficacy quantitation fluorescence of EGFP was collected at 525 nm (FL1). For cytotoxicity measurements BD Via-Probe kit was employed. This reagent was used for dead cell exclusion. Five microliters of the kit was added to each sample and after 10 min of incubation fluorescence correspondent to dead cells was measured at 650 nm (FL3).

2.12 Statistical analysis

Results are reported as means (S.D. = standard deviation). Statistical analysis was made with SPSS 14.0 for Windows® (SPSS®, Chicago, USA). Normal distribution of samples was assessed by the Shapiro–Wilk's test, and homogeneity of the variance by the Levene's test. The statistical analysis between different groups was determined with an ANOVA test. Differences were considered statistically significant if $p < 0.05$.

3. RESULTS

3.1 Characterization of SLNs

Table 1 summarizes particle size, zeta potential and polydispersity indexes of SLNs composed by different amounts of DOTAP and Tween-80. The particle size ranged from 340 to 373 nm and the zeta potential was positive, around +45 mV, for all formulations.

Polydispersity indexes were 0.4, 0.2 and 0.3 for SLNs composed by DOTAP 0.4% Tween-80 0.1%, DOTAP 0.3% Tween-80 0.2% and DOTAP 0.3% Tween-80 0.1%, respectively. No statistically significant differences ($p > 0.05$) were observed between the formulations.

Table 1. Composition and physicochemical characterization of SLN formulations.

Cationic lipid	Tween-80	Size (nm)	Zeta potential (mV)	Polydispersity Index
DOTAP 0.4%	0.1%	339.1 (12.4)	+42.9 (1.3)	0.4 (0.1)
DOTAP 0.3%	0.2%	373.2 (42.9)	+48.1 (5.6)	0.2 (0.2)
DOTAP 0.3%	0.1%	367.3 (129.6)	+49.8 (5.9)	0.3 (0.1)

Mean (S.D. = Standard deviation) (n=3). The matrix lipid was Precirol® ATO 5 at 5% in all formulations.

The atomic force microscopy (AFM) images of SLNs indicated that nanoparticles were spherical. The image in Figure 1A corresponds to the visualization of a batch of SLNs.

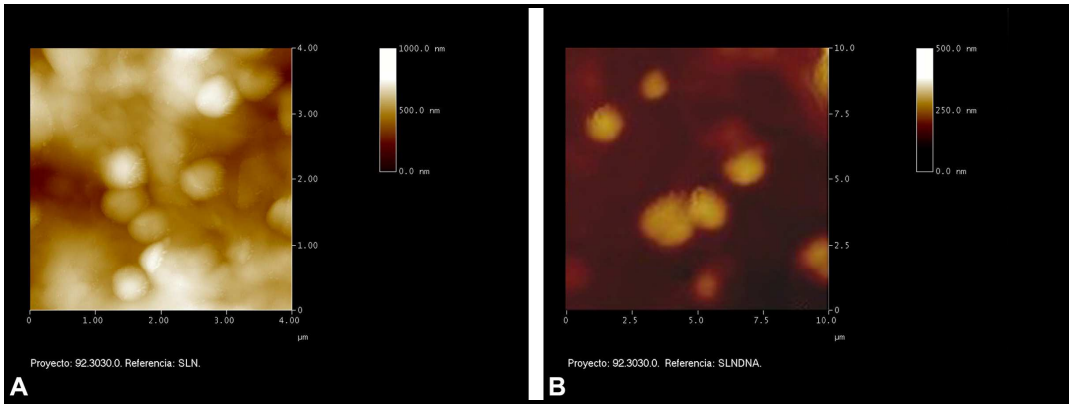

Figure 1. Image of SLNs (A) and SLN–DNA complexes (B) taken by atomic force microscopy. SLNs were composed by DOTAP 0.4% and Tween-80 0.1%, and SLN–DNA complexes were prepared at DOTAP:DNA ratio 5:1 (w/w).

3.2 Characterization of SLN-DNA complexes

SLN–DNA complexes for an extensive characterization were prepared with the SLNs composed by DOTAP 0.4% and Tween-80 0.1%.

As can be seen in Table 2, the size of the complexes obtained at the DOTAP:DNA ratios from 15:1 to 4:1 decreased when comparing with their corresponding SLNs (Table 1), whereas a size increase took place with the ratio 3:1 or smaller (bigger than 1 μm). No significant differences between the sizes of the complexes at ratios 15:1 to 4:1 were detected ($p > 0.05$). Polydispersity indexes were higher than those obtained with SLNs.

The zeta potential (Table 2) was highly positive for DOTAP:DNA ratios 15:1 to 4:1, ranging from +30 to +40 mV. However, when DOTAP:DNA ratio was 3:1 or lower zeta potential became negative.

Table 2. Physicochemical characterization of SLN-DNA complexes.

DOTAP:DNA ratio (w/w)	Size (nm)	Zeta potential (mV)	Polydispersity Index
15:1	258.5 (23.1)	+40.6 (5.6)	0.5 (0.3)
10:1	216.7 (44.2)	+46.6 (4.4)	0.7 (0.3)
7:1	260.1 (18.7)	+38.9 (7.1)	0.7 (0.2)
6:1	255.9 (61.7)	+35.3 (2.6)	0.7 (0.2)
5:1	249.2 (25.3)	+36.5 (2.8)	0.4 (0.3)
4:1	240.4 (10.9)	+32.4 (0.9)	0.7 (0.1)
3:1	> 1 μm	-11.9 (5.2)	-
2.5:1	> 1 μm	-23.7 (2.9)	-
1:1	> 1 μm	-37.1 (1.9)	-

SLNs were composed by DOTAP 0.4% and Tween 80 0.1%. The size of the corresponding SLNs was 339.1 (12.4). Mean (S.D. = standard deviation) (n=3).

Figure 2 shows the gel electrophoresis resulting from the binding efficiency assay carried out with the different DOTAP:DNA ratios used in the elaboration of the complexes. Lane 1 corresponds to free DNA. On lanes 2–7 (ratios from 15:1 to 4:1) there was no band corresponding to free DNA, which means that all DNA was bound to the SLNs. Ratios 3:1, 2.5:1 and 1:1 (lanes 8, 9 and 10, respectively) revealed bands indicating that SLNs were not capable to bind all DNA. These results are in agreement with those obtained with the PicoGreen® assay, which showed that DOTAP:DNA ratios equal or higher than 4:1 presented a binding efficiency of 100%, whereas when this ratio was 3:1 or smaller the binding efficiency decreased.

Figure 2. Binding efficiency of DNA with SLNs at different DOTAP:DNA ratios (w/w) studied by agarose gel electrophoresis. Lane 1= free DNA; lane 2 = 15:1; lane 3 = 10:1; lane 4 = 7:1; lane 5 = 6:1; lane 6 = 5:1; lane 7 = 4:1; lane 8 = 3:1; lane 9 = 2.5:1; lane 10 = 1:1.

3.3 "*In vitro*" resistance against DNase I

Figure 3 features the gel electrophoresis with the results of resistance of DNA bound to SLNs against the attack of DNase I. Lanes 1 and 2 correspond to non-treated free DNA and DNase I treated free DNA, respectively. No band on lane 2 is observed because free DNA was totally digested by the enzyme. The presence of bands on lanes 3–8 demonstrated that SLN–DNA complexes prepared at DOTAP:DNA ratios from 15:1 to 4:1 were able to protect DNA. DOTAP:DNA ratios equal or below 3:1 failed to prevent nuclease digestion of the DNA (lanes 9–11).

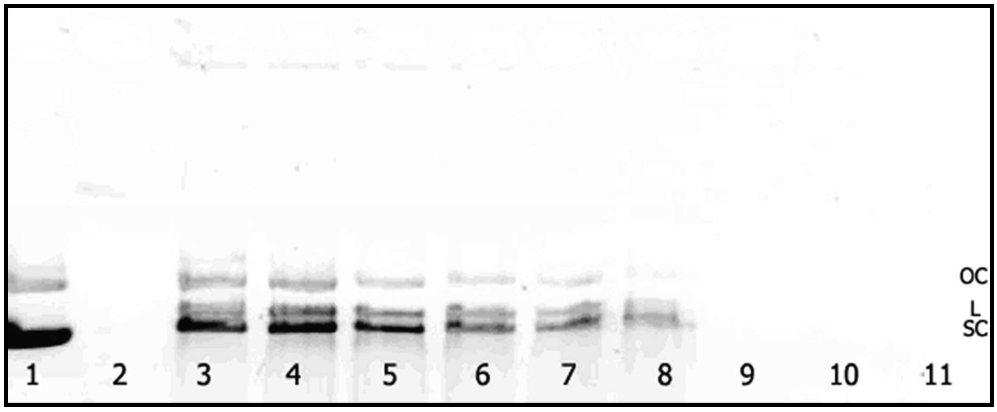

Figure 3. Protection of DNA by SLNs from DNase digestion at different DOTAP:DNA ratios (w/w) visualized by agarose gel electrophoresis. SLN–DNA complexes were treated with DNase I. Lane 1 = not treated free DNA; lane 2 = DNase-treated free DNA; lane 3 = 15:1; lane 4 = 10:1; lane 5 = 7:1; lane 6 = 6:1; lane 7 = 5:1; lane 8 = 4:1; lane 9 = 3:1; lane 10 = 2.5:1; lane 11 = 1:1; OC: open circular form; L: lineal form; SC: supercoiled form.

The agarose gel electrophoresis also allowed us to detect the presence of different DNA topology forms after the treatment with DNase I. Non-treated free DNA (lane 1), which was not in contact with the DNase, presented only two bands. The lower band corresponds to the supercoiled form (SC) and the upper band to the open circular form (OC). When the SLN–DNA complexes were treated with DNase I another intermediate band appeared, which corresponds to the linear form (L).

3.4 Transfection "*in vitro*"

The highest transfection activity (Figure 4A) was obtained with SLNs composed by DOTAP 0.4% and Tween-80 0.1%: 14.8% cells produced green fluorescent protein (EGFP). This transfection level was statistically higher ($p < 0.05$) than the percentage of transfected cells obtained with the SLNs composed by DOTAP 0.3% and Tween-80 0.2% or DOTAP 0.3% and Tween-80 0.1% (5.9% and 4.0% transfected cells, respectively). No statistically significant differences appeared between these two formulations ($p > 0.05$).

Figure 5A shows green fluorescence due to the transfection of cells with SLN–DNA complexes composed by DOTAP 0.4% and Tween-80 0.1%, at DOTAP:DNA ratio 5:1, and Figure 5B the correspondent phase contrast image.

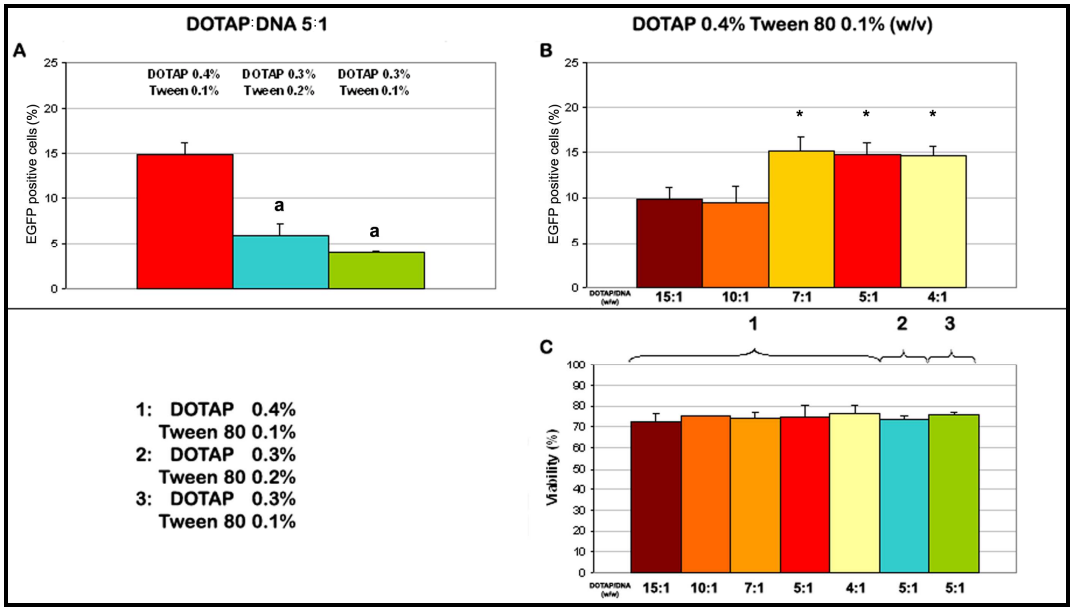

Figure 4. Transfection activity and cell viability of the different formulations assayed: (A) influence of SLNs composition on transfection activity; (B) influence of DOTAP:DNA ratio on transfection activity; (C) percentage of alive cells after the treatment with the different formulations. $^{a}p < 0.05$ against to DOTAP 0.4%, Tween-80 0.1%. $^{*}p < 0.05$ against to DOTAP:DNA ratios 15:1 and 10:1.

In order to study the influence of the ratio SLN:DNA on the transfection ability we chose the formulation composed by DOTAP 0.4% and Tween-80 0.1% because it was the formulation with the highest transfection capacity. DOTAP:DNA ratios employed were 15:1, 10:1, 7:1, 5:1 and 4:1.

As can be seen in Figure 4B, the complexes prepared at the highest ratios (15:1 and 10:1) provided smaller transfection levels ($p < 0.05$), around 9.5% EGFP-positive cells, than the complexes prepared using ratios 7:1, 5:1 and 4:1, which transfected about 15%

of the cells in culture. No significant differences were detected between these three formulations ($p > 0.05$).

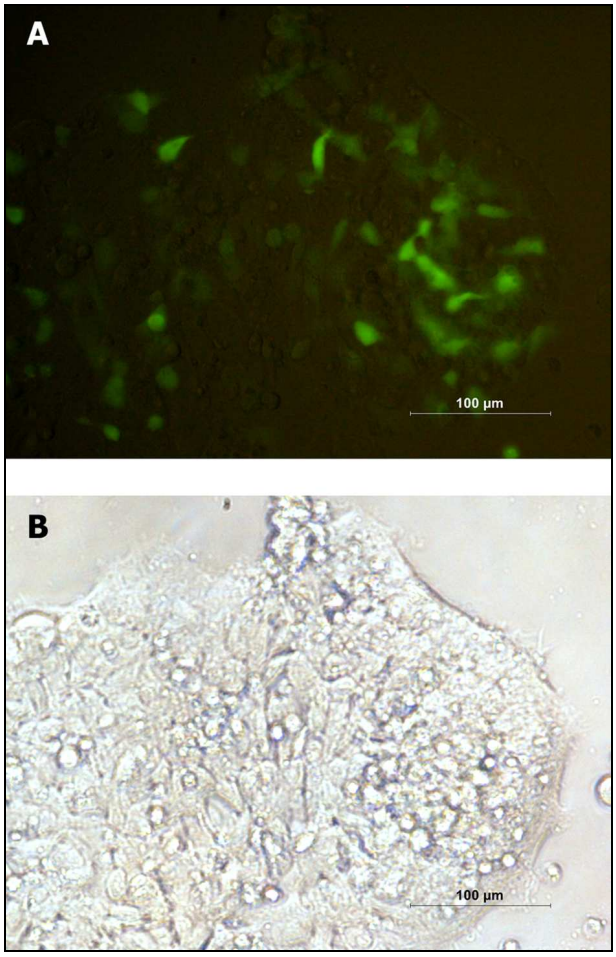

Figure 5. EGFP-positive cells obtained with SLN–DNA complexes composed by DOTAP 0.4% and Tween-80 0.1%, at DOTAP:DNA ratio 5:1 (w/w) observed by inverted fluorescent microscopy (20× objective): (A) fluorescence image; (B) phase contrast image.

3.5 Cytotoxicity of the different formulations on HEK293 cell culture

In order to evaluate the effect of the formulations on cell viability, the percentage of dead cells was determined by flow cytometry. Figure 4C presents these results. The viability was near 75% for both the cells treated with transfection systems and the non-treated cells ($p > 0.05$).

After 1-month storage of the SLNs at 4 ºC the transfection levels and the cell viability obtained with these systems were maintained (data not shown).

4. DISCUSSION

In spite of the advantages of SLNs over liposomes, mainly in relation to their stability and manufacture processes, very few reports about the use of SLNs in gene delivery[3,4,5,6,7] have been published.

We have prepared nanoparticles with Precirol® ATO 5, DOTAP and Tween-80, and we evaluated their transfection capacity. Furthermore, we evaluated the influence of the DOTAP:DNA ratio on the transfection activity of the SLN–DNA complexes.

Cationic lipids are needed for the preparation of SLNs used on gene therapy because of their surfactant activity and their positive charge. The surfactant activity is necessary to obtain the initial emulsion, and the positive charge to provide the superficial charge to the SLNs. The positive superficial charge of the SLNs is needed for their further interaction with negative charged DNA to form SLN–DNA complexes. However, cationic lipids can be toxic on repeated use and can induce inflammatory reactions "*in vivo*"[15]. We prepared SLNs with one of the most commonly used cationic lipids in gene therapy, DOTAP. The minimum proportion necessary to form a stable emulsion was 1%. Due to the risk of toxicity of cationic tensioactives we decreased the proportion of DOTAP in the formulations thanks to the addition of Tween 80. As we mentioned above this tensioactive has flattering properties for gene therapy in terms of formulation and transfection activity.

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15 Han So, Mahato RI, Sung YK, Kim SW. Development of biomaterials for gene therapy. Mol Ther. 2000; 2: 302-317.

Once the SLNs were synthesized, we proceeded to prepare the SLN–DNA complexes. As can be seen in Figure 1 the spherical shape was maintained. However, the size of the complexes (Table 2) was smaller than the size of the correspondent SLNs when DOTAP:DNA ratios from 15:1 to 4:1 were used (339 nm versus approximately 250 nm). The reduction in size is due to the DNA compactation. At the minor ratios (3:1, 2.5:1 and 1:1) the size increased because the SLNs lost the DNA binding and condensation capacity. We have observed that the degree of DNA condensation increases when increasing the cationic lipid:DNA ratio. Similar results have been obtained by Faneca et al.[13] Mahato et al.[16] and Ferrari et al.[17].

The zeta potential (Table 2) was highly positive, between +30 and +40 mV, for the highest DOTAP:DNA ratios (from 15:1 to 4:1), but for the minor ratios (3:1, 2.5:1 and 1:1) the zeta potential became negative. The smaller the DOTAP:DNA ratio is, the lower DNA condensation is provided by the complexes, which allows for the exposure of the DNA-negative charges. For gene therapy the positive charge of the non-viral systems is advantageous because it facilitates the interaction with the negative charged cell surface and the cell entry. Elouahabi and Ruysschaert[18] have postulated that this positive charge could also improve the entry in the cell facilitating the invagination of the cell plasma membrane and inducing the early steps of the endocytosis process. Clathrin-mediated

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16 Mahato RI, Kawabata K, Nomura T, Takakura Y, Hashida M. Physicochemical and pharmacokinetic characteristics of plasmid DNA cationic liposome complexes. J Pharm Sci. 1995; 84: 1267-1271.

17 Ferrari ME, Nguyen CM, Zelphati O, Tsai YL, Felgner PL. Analytical methods for the characterization of cationic lipid nucleic acid complexes. Hum Gene Ther. 1998; 9: 341-351.

18 Elouahabi A, Ruysschaert JM. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol Ther. 2005; 11: 336-347.

endocytosis is the main entry process for the cationic lipid formulations[19], but fusion is also possible. We have prepared formulations with positive superficial charge, which improves endocytic pathways because, when the systems enter by fusion, part of the DNA can be released to the medium and not to the cytoplasm[20].

The binding efficiency assay showed that DOTAP:DNA ratios 4:1 or higher are necessary to bind DNA completely. When DOTAP:DNA ratios from 3:1 to 1:1 were employed the binding efficiency decreased and it was reduced to almost half at DOTAP:DNA ratio 1:1.

An important advantage of non-viral systems in gene therapy is their capacity to protect DNA from components of the medium, and fundamentally from DNases digestion. In order to study the protection capacity of the SLNs, we put SLN–DNA complexes prepared at different DOTAP:DNA ratios in contact with DNase I during 30 min at 37 ºC, and we analyzed the integrity of the DNA by agarose gel electrophoresis (Figure 3). Results showed that SLNs were able to protect DNA only at DOTAP:DNA ratios 4:1 or higher. However, the intensity of the bands decreased as the DOTAP:DNA ratio decreased, which indicates that protection of the genes from DNases depends on condensation degree. When the condensation decreases DNA exposition to components in medium increases, and the digestion by enzymes is easier.

Once the formulations possessed the technologically suitable characteristics for the desired application, the next step was their evaluation "*in vitro*".

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19 Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther. 2005; 12: 468-474.

20 Elouahabi A, Thiry M, Pector V, Ruysschaert JM, Vandenbranden M. Calorimetry of cationic liposome–DNA complex and intracellular visualization of the complexes. Methods Enzymol. 2003; 373: 312-332.

On the basis of the results mentioned above, for transfection assays we worked with DOTAP:DNA ratios from 15:1 to 4:1: those which bound all DNA, provided high positive surface charge and protected DNA from enzyme degradation.

Regarding the transfection activity of the different SLN–DNA formulations, those composed by the highest proportion of DOTAP, 0.4% (w/v), provided the highest transfection levels (Figure 4A), whereas the transfection obtained from those containing a lower proportion of DOTAP, 0.3% (w/v), were statistically smaller, independently of the amount of Tween-80. Although the transfection activity "*in vitro*" was influenced by the cationic lipid proportion, and not by the Tween-80 concentration, this non-ionic surfactant determined the size and morphology of SLNs because it was necessary to form a stable and homogeneous emulsion employing a lower DOTAP amount, and thus, reduce the formulation toxicity.

The transfection efficiency of the SLNs was also influenced by the DOTAP:DNA ratio. At ratios 15:1 and 10:1 the transfection levels were significantly smaller than those obtained with the ratios 7:1, 5:1 and 4:1 (Figure 4B). These differences could be explained by the condensation degree of the DNA. Condensation is necessary to facilitate the mobility of DNA molecules, which is limited by their large size. Furthermore, it is interesting that formulations are able to bind and condensate DNA, providing positive charged complexes which protect DNA from external agents. However, if condensation is excessive, the DNA release from the complexes is more difficult and transfection could be limited. In fact, the release of DNA from the complexes may be one of the most crucial

steps determining the optimal ratio for cationic lipid system-mediated transfection[21]. In order to have an indication about the release of DNA from our SLNs based complexes, we quantified free DNA in the culture medium of cells after 4 h in contact with the complexes. For ratios 15:1, 10:1 and 7:1 about 16% of the DNA incorporated into the complexes and added to the culture cell was free in the medium, compared to 37% detected at the minor ratios (5:1 and 4:1). These results fit in with those observed in Figure 3, where the different bands intensity could suggest that DNA condensation is higher at ratios 15:1 to 7:1 than at 5:1 and 4:1.

Faneca et al.[13] studied the transfection capacity of cationic liposomes at different charge ratios (+:−) and obtained lower gene expression levels with complexes prepared at charges ratio 8:1 (+:−) as compared to 4:1 (+:−) complexes. They attributed that difference to the excessive condensation at charge ratio 8:1 (+:−), which made the dissociation of DNA from the liposomes difficult. Our results coincide with those reported in their work.

Another important aspect to be considered for the design of this kind of formulations is DNA topology. DNA can feature three forms: supercoiled (SC), open circular (OC) and linear (L). SC-DNA has been reported in the literature to be the most bioactive form[22,23]. Figure 3, obtained from the DNase I protection study, shows the presence of the L-form

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21 Sakurai F, Inoue R, Nishino Y, Okuda A, Matsumoto O, Taga T, et al. Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression. J Control Release. 2000; 66: 255-269.

22 Middaugh CR, Evans RK, Montgomery DL, Casimiro DR. Analysis of plasmid DNA from a pharmaceutical perspective. J Pharm Sci. 1998; 87: 130-146.

23 Remaut K, Sanders NN, Fayazpour F, Demeester J, De Smedt SC. Influence of plasmid DNA topology on the transfection properties of dotap/dope lipoplexes. J Control Release. 2006; 115: 335-343.

after the treatment of the complexes with that enzyme. DNase I turns the SC-DNA, which is the DNA topology with the most transfection capacity, into OC by cutting one of the DNA double strands, and in a successive cut, it breaks the OC- to the L-DNA[24]. Thus, as the formulations with higher DOTAP:DNA ratio are the ones that better protect the DNA from DNases, they might be the most convenient for transfection from a point of view of DNA topology.

Therefore, DNA condensation is a crucial factor which determines the transfection capacity of SLNs, because it influences the superficial charge of the complexes and thus cell entry, DNA delivery from nanoparticles, gene protection from DNAses and hence DNA topology. An optimal DNA condensation must be achieved when designing non-viral vectors. Complexes must have enough DNA condensation capacity to create an equilibrium between those three factors to obtain good transfection levels.

We have also shown that SLN-complexes composed by Precirol® ATO 5, DOTAP and Tween-80 do not decrease cell viability and present good stability properties after the storage of the SLNs 1 month at 4º C (data not shown).

5. CONCLUSIONS

In conclusion, this study shows the potential of solid lipid nanoparticles (SLNs) as non-viral vectors for gene therapy and the main factors which can determine the efficacy of these systems. The "*in vitro*" transfection levels provided by the formulations developed are conditioned mainly by their DNA condensation capacity. There must be an equilibrium between the gene protection degree, the binding forces of DNA to SLNs, and the DNA

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24 Sanders NN, De Smedt SC, Demeester J. In: McGrath BM and Walsh G. (Eds.) Therapeutic Enzyms. 2006; Taylor & Fracis Group, Boca Raton, pp. 97-116.

topology. This equilibrium is determined by cationic lipid:DNA ratio and it must be optimized with every new formulation.

AKNOWLEDGMENTS

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SOLID LIPID NANOPARTICLES FOR RETINAL GENE THERAPY: TRANSFECTION AND INTRACELLULAR TRAFFICKING IN RPE CELLS

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ABSTRACT

Retinal pigment epithelial (RPE) cells are usually employed to study DNA systems for diseases related to problems in the retina. Solid lipid nanoparticles (SLNs) have been shown to be useful non-viral vectors for gene therapy. The objective of this work was to evaluate the transfection capacity of SLNs in the human retinal pigment epithelial established cell line (ARPE-19) in order to elucidate the potential application of this vector in the treatment of retinal diseases. Results showed a lower transfection level of SLNs in ARPE-19 cells than in HEK293 (2.5% vs. 14.9% EGFP positive cells at 72 h post-transfection). Trafficking studies revealed a delay in cell uptake of the vectors in ARPE-19 cells. Differences in internalization process into the two cell lines studied explain, in part, the difference in the gene expression. The clathrin-mediated endocytosis in ARPE-19 cells directs the solid lipid nanoparticles to lysosomes; moreover, the low division rate of this cell line hampers the entrance of DNA into the nucleus. The knowledge of intracellular trafficking is very useful in order to design more efficient vectors taking into account the characteristics of the specific cell line to be transfected.

Keywords: Solid lipid nanoparticles; Transfection; Intracellular trafficking; ARPE-19 cells; Caveolae; Clathrin

1. INTRODUCTION

Nowadays multiple diseases may be the object of gene therapy, from monogenic diseases such as cystic fibrosis[1] to more complex diseases such as cancer[2], and specifically those related to ocular disorders are attracting increasing interest. The eye is a promising organ for gene therapy because of its well-defined anatomy, immunoprivilege and accessibility. Furthermore, as the media is transparent, the gene transfer process can be easily seen[3]. Gene therapy with viral vectors has been proved to be an efficient treatment for some retina related diseases, such as Leber congenital amaurosis[4,5] or X-linked juvenile retinoschisis[6]. Retinal pigment epithelial (RPE) cells are usually employed "*in vitro*"[7,8,9,10,11] as a tool to evaluate new therapeutic strategies in gene therapy for retinal diseases.

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1 Davies JC. Gene and cell therapy for cystic fibrosis. Paediatr Respir Rev. 2006; 7: S163–S165.

2 Brandwijk RJMG, Griffioen AW, Thijssen VLJL. Targeted gene-delivery strategies for angiostatic cancer treatment. Trends Mol Med. 2007; 13: 200–209.

3 Liu XY, Brandt CR, Rasmussen CA, Kaufman PL. Ocular drug delivery: molecules, cells, and genes. Can J Ophthalmol. 2007; 42: 447–454.

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6 Min SH, Molday LL, Seeliger MW, Dinculescu A, Timmers AM, Janssen A, et al. Prolonged recovery of retinal structure/function after gene therapy in an Rs1h deficient mouse model of X-linked juvenile retinoschisis. Mol Ther. 2005; 12: 644–651.

7 Chaum E, Hatton MP, Stein G. Polyplex-mediated gene transfer into human retinal pigment epithelial cells in vitro. J Cell Biochem. 2000; 76: 153–160.

8 Doi K, Hargitai J, Kong J, Tsang SH, Wheatley M, Chang S, et al. Lentiviral transduction of green fluorescent protein in retinal epithelium: evidence of rejection. Vis Res. 2002; 42: 551–558.

Non-viral vectors are being extensively studied in gene therapy as an attractive alternative to viral-based systems due to the reduction of risks such as oncogenicity or immunogenicity, their easier manufacture and the absence of DNA size limit. However, their clinical use requires improvement in terms of effectiveness, which depends not only on the administration system but also on the targeted cells.

It is known that transfection is conditioned by the entry and posterior intracellular trafficking of the vectors and these processes are cell line dependent[12,13]. Figure 1 illustrates the barriers that DNA delivery systems have to overcome during the trafficking to the nucleus before the synthesis of the encoded protein. First, vectors have to bind to cell surface, which occurs by electrostatic interactions between the positively charged systems and the negative charges of the cell membrane. The next step consists in the entry into the cell, with endocytosis as the main process postulated[14,15,16]. Once inside the

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9 Pitkanen L, Pelkonen J, Ruponen M, Ronkko S, Urtti A. Neural retina limits the nonviral gene transfer to retinal pigment epithelium in an in vitro bovine eye model. AAPS J. 2004; 6.

10 Mannisto M, Ronkko S, Matto M, Honkakoski P, Hyttinen M, Pelkonen J, et al. The role of cell cycle on polyplex-mediated gene transfer into a retinal pigment epithelial cell line. J Gene Med. 2005; 7: 466–476.

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14 Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene-transfer by a cationic lipid. J Biol Chem. 1995; 270: 18997–19007.

15 Zuhorn IS, Kalicharan R, Hoekstra D. Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. J Biol Chem. 2002; 277: 18021–18028.

cell, the DNA has to cross the nuclear envelope to reach the cellular machinery for protein synthesis.

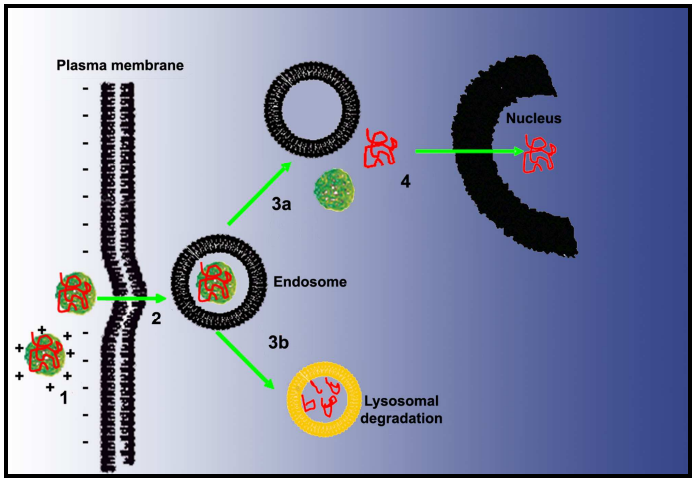

Figure 1. Trafficking of non-viral vectors during the transfection process. (1) Binding to plasma membrane due to electrostatic interactions between the positive charged vectors and the negative charges of the plasma membrane. (2) Entry into the cell: endocytosis has been established as the main entry mechanism. (3a) Displacement of the endosomes to the prosimity of the nucleus and release of the vectors to the cytoplasm. (3b) The exit of vectors from endosomes has to take place before the fusion with lysosomes, which possess enzymes with ability to digest DNA. (4) Entry of DNA into the nucleus. This step can occur through pores in nuclear envelope or during mitosis, when the nuclear membrane is disrupted.

Cationic lipid-based systems formulated as liposomes, solid lipid nanoparticles (SLNs) or emulsions[17,18,19,20,21], are included in the group of non-viral systems for DNA delivery. In the last years SLNs have attempted a big development as drug delivery systems, but

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16 Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther. 2005; 12: 468–474.

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20 Tabatt K, Kneuer C, Sameti M, Olbrich C, Muller RH, Lehr CM, et al. Transfection with different colloidal systems: comparison of solid lipid nanoparticles and liposomes. J Control Release. 2004; 97: 321–332.

21 Salvati A, Ciani L, Ristori S, Martini G, Masi A, Arcangeli A. Physicochemical characterization and transfection efficacy of cationic liposomes containing the pEGFP plasmid. Biophys Chem. 2006; 121: 21–29.

there are still few papers about their use in gene therapy. However, the publications about the use of cationic liposomes in this field are numerous, although by now no formulation has been marketed for nucleic acid delivery. From the point of view of application, SLNs have good stability[22], which facilitates the industrial elaboration and the manipulation for different processes such as lyophilization and they can be a promising alternative to the liposomes. We have already shown that SLNs composed by Precirol® ATO 5 as core lipid, DOTAP as cationic lipid and Tween 80 as tensioactive transfect the plasmid encoding the enhanced green fluorescent protein (pCMS-EGFP) in Human Embryonic Kidney (HEK293) culture cells[23]. This cell line is usually employed in "*in vitro*" transfection studies because its culture conditions and manipulation are optimized, and it is one of the best transfected cell lines, which makes it a good model for "*in vitro*" transfection studies. The objective of this work was to evaluate the transfection capacity of SLNs in the human retinal pigment epithelial established cell line (ARPE-19) in order to elucidate the potential application of this vector in the treatment of retinal diseases. Due to the importance of the cell uptake and the intracellular behaviour of the vectors we have studied the intracellular trafficking of the SLNs in ARPE-19 and HEK293 cells.

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2. MATERIALS AND METHODS

2.1 Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). *N*-[1-(2,3-Dioleoyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), antibiotic/antimycotic and Nile Red were purchased from Sigma–Aldrich (Madrid, Spain). Tween-80 was provided by Vencaser (Bilbao, Spain) and dichloromethane by Panreac (Barcelona, Spain).

Plasmid pCMS-EGFP encoding the enhanced green fluorescent protein (EGFP) was purchased from BD Biosciences Clontech (Palo Alto, US) and amplified by Dro Biosystems S.L. (San Sebastián, Spain). The labelling of the plasmid pCMS-EGFP with ethidium monoazide (EMA) was also carried out by Dro Biosystems S.L. (San Sebastián, Spain).

Hoechst 33258, AlexaFluor488-Cholera toxin and AlexaFluor488-Transferrin were provided by Molecular Probes (Barcelona, Spain), and Fluoromount G from SouthernBiotech (Coultek, Spain).

Cell culture reagents were purchased from LGC Promochem (Barcelona, Spain). Antibiotic Normocin™ was acquired from InvivoGen (San Diego, CA).

2.2 Preparation of SLN-DNA vectors

The SLNs were produced by a solvent emulsification/evaporation technique[24]. The lipid Precirol® ATO 5 was dissolved in the organic solvent dichloromethane (5%, w/v), and then emulsified in an aqueous phase containing the cationic lipid DOTAP (0.4%, w/v) and

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the surfactant Tween-80 (0.1%, w/v). The organic phase:aqueous phase ratio was 1:5. The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) during 30 s at 50W. The organic solvent then was removed from the emulsion by evaporation using a magnetic agitator for 45 min followed by vacuum conditions for 15 min. Upon dichloromethane evaporation SLNs suspension was formed by precipitation of the Precirol® ATO 5 in the aqueous medium. Finally, SLNs were washed by centrifugation (3000 rpm, 20min, 3 times) using the Amicon® Ultra centrifugal filters (Millipore, Madrid, Spain).

To prepare SLN-DNA complexes, a solution of pCMS-EGFP plasmid was mixed with the SLNs suspension. The ratio SLN:DNA, expressed as DOTAP:DNA ratio (w/w), was 5:1.

In order to analyze the trafficking of the vectors, SLNs were labelled with Nile Red (λ= 590 nm), and the plasmid pCMS-EGFP with ethidium monoazide (EMA; λ = 625 nm). The label of the SLNs was based on a method reported by Borgia et al.[25] to prepare Nile Red loading nanoparticular systems. SLNs were prepared by the emulsification/evaporation technique described above, incorporating the Nile Red in the dichloromethane. The plasmid was labelled according to the procedure described by Ruponen et al.[26] One volume of ethidium monoazide bromide (Sigma–Aldrich) at 10 μg/ml in water was added to one volume of pCMS-EGFP at 400 μg/ml in water and the mixture was incubated for 1

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25 Borgia SL, Regehly M, Sivaramakrishnan R, Mehnert W, Korting HC, Danker K, et al. Lipid nanoparticles for skin penetration enhancement-correlation to drug localization within the particle matrix as determined by fluorescence and parelectric spectroscopy. J Control Release. 2005; 110: 151–163.

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h at room temperature in darkness. After the incubation period, the solution was exposed to UV light for 5 min. Gel filtration on NAP-25 Column (GE Healthcare) was used to purify the labelled DNA from free EMA. To remove intercalated but not covalently bound EMA, CsCl was added to a concentration of 1.1 g/ml and was gently mixed until it dissolved. Plasmid was extracted with CsCl-saturated isopropanol. The isopropanol washing step was repeated until the upper phase appeared clear. CsCl was removed by dialysis against water, and the labelled EMA-pCMS-EGFP plasmid was recovered with isopropanol precipitation.

2.3 Size and zeta potential measurements

The sizes of SLNs and SLN-DNA complexes were determined by photon correlation spectroscopy (PCS). Zeta potential was measured by laser doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in NaCl 0.1mM.

2.4 Cell culture and transfection protocol

"*In vitro*" assays were performed with two different cell lines: Human Embryonic Kidney (HEK293) cell line and Human Retinal Pigmented Epithelial (ARPE-19) cell line, obtained from the American Type Culture Collection (ATCC).

HEK293 cells were maintained in Eagle's Minimal Essential Medium with Earle's BSS and 2mM L-glutamine (EMEM) supplemented with 10% heat-inactivated horse serum and 1% antibiotic/antimycotic. Cells were incubated at 37ºC with 5% CO_2 in air and subcultured every 2–3 days using trypsin/EDTA. For transfection HEK293 cells were seeded on 24 well plates at a density of 150,000 cells per well and allowed to adhere overnight.

ARPE-19 cells were maintained in Dulbecco's Modified Eagle's Medium/Han's Nutrient Mixture F-12 (1:1) medium (D-MEM/F-12) supplemented with 10% heat-inactivated fetal calf serum and 1% antibiotic solution Normocin™. Cells were incubated at 37ºC with 5% CO_2 in air and subcultured every 2–3 days using trypsin/EDTA. For transfection ARPE-19 cells were seeded on 12 well plates at a density of 30,000 cells per well and allowed to adhere overnight.

Seventy-five microliters of the vectors solution diluted in HBS (2.5 μg DNA) were added to each well, and cells were incubated with the vectors for 4 h at 37 ºC. After that time the medium containing the complexes in the wells was diluted with 1ml of complete medium and cells were allowed to grow for further 72 h.

2.5 Flow cytometry-mediated analysis of transfection efficacy

At the end of the incubation period, the cells were washed once with 300 μl of PBS and were detached with 300 μl of 0.05% trypsin/EDTA. Then the cells were centrifuged at 1500*g* and the supernatant was discarded. Cells were re-suspended with PBS and directly introduced to a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, USA). Fluorescence of EGFP was collected at 525 nm (FL1). For each sample 10,000 events were collected.

2.6 Cellular uptake of non-viral vectors

Entry of vectors to the cells was studied quantitatively by flow cytometry and qualitatively by confocal laser scanning microscopy (CLSM). For this purpose SLNs were labelled with the fluorescent dye Nile Red (λ = 590 nm).

Cells incorporating vectors were quantified by flow cytometry at 650 nm (FL3) after detachment from plates at different time points. For each sample 10,000 events were collected.

For the CLSM study, cells were seeded in coverslips containing plates and treated with the vectors. At different incubation times, the medium was removed and cells were washed with PBS and fixed with paraformaldehyde 4%. We had previously checked that paraformaldehyde did not interact with the fluorescence of NileRed or EGFP. Preparations were mounted on Fluoromount G and after air-drying images were obtained with an Olympus Fluoview FV500 confocal microscope using sequential acquisition to avoid overlapping of fluorescent emission spectra. CLSM images were captured in the General Service of Analytical Microscopy and High Resolution in Biomedicine of the University of the Basque Country (UPV-EHU).

2.7 Co-localization assay

In order to identify the specific endocytic processes involved in the non-viral systems internalization cells were seeded in coverslips and co-incubated for 1 h with Nile Red labelled vectors and either AlexaFluor488-Cholera toxin (10 μg/ml) or AlexaFluor488-Transferrin (50 μg/ml), which are markers for caveolae-mediated endocytosis and clathrin-mediated endocytosis, respectively. Cell fixation and images acquisition were performed as described in Section 2.6.

2.8 Detection of intracellular EMA-labelled DNA by fluorescence microscopy

In order to evaluate the trafficking of DNA in the cytoplasm, cells were seeded in culture plates and treated with vectors containing pCMS-EGFP labelled with ethidium

monoazide (EMA). Prior to the observation of the samples through the microscope, nucleuses were labelled with Hoechst 33258. Images were captured with an inverted microscopy equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon). Observations and image captures were performed using a 20× objective.

2.9 Statistical analysis

Results are reported as means (SD = standard deviation). The statistical analysis was carried out with SPSS 14.0 for Windows® (SPSS®, Chicago, USA). Normal distribution of samples was assessed by the Shapiro–Wilk's test, and homogeneity of the variance by the Levene's test. The statistical analysis between HEK293 and ARPE-19 cells was determined with a Student's t-test. Differences were considered statistically significant if $p < 0.05$.

3. RESULTS

3.1 Characterization of vectors: size and zeta potential

Table 1 summarizes the size and zeta potential of the vectors employed in this work. The positive charge allows vectors to bind to the negative charged cell surface and particle size influences the mechanism of cell internalization[27]. Results show that neither the Nile Red nor the ethidium monoazide (EMA) induced changes in particle size or zeta potential of SLN-DNA vectors ($p > 0.05$).

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27 Rejman J, Oberle V, Zuhorn IS, Hoekstra D. 2004. Size-dependent internalization of particles via the pathways of clathrin-and caveolae-mediated endocytosis. Biochem J. 2004; 377: 159–169.

Table 1. Size and zeta potential of SLN-DNA vectors

	SLN-EGFP	Nile-EGFP	SLN-EMA
Size (nm)	281 (69)	269 (69)	222 (5)
Z potential (mV)	+30 (1)	+29 (1)	+30 (2)

SLN-EGFP: vectors composed by non-labelled SLNs and non-labelled plasmid. Nile-EGFP: vectors composed by NileRed-labelled SLNs and non-labelled plasmid. SLN-EMA: vectors composed by non-labelled SLNs and EMA-labelled plasmid. SLN:DNA ratio 5:1 (w/w). Mean (S.D. = standard deviation) (n=3).

3.2 Transfection levels in culture cells

The transfection capacity of SLN-DNA vectors in HEK293 and ARPE-19 cells was evaluated. The percentages of cells expressing EGFP were measured at different times from 0.5 to 72 h after the addition of the vectors. Bars in Figure 2 represent those levels. Results show that in HEK293 culture cells green fluorescence was detected from 24 h, while in ARPE-19 cells green fluorescence was only detected at 72 h. Moreover, transfection levels in HEK293 (14.9% EGFP positive cells) were higher ($p < 0.01$) than in ARPE-19 cells (2.5% EGFP positive cells).

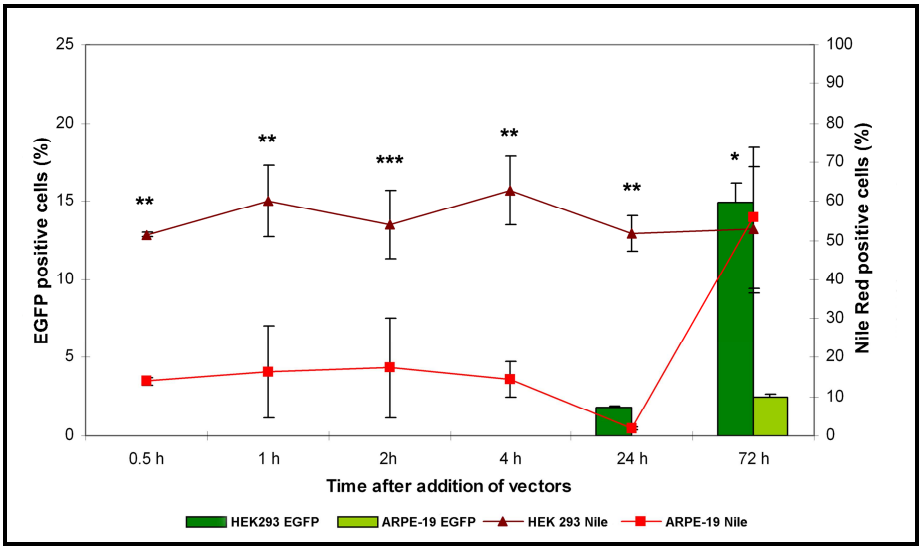

Figure 2. Transfection and uptake of non-viral vectors by HEK293 and ARPE-19 cells. Bars represent the percentage of transfected cells. Lines represent the percentage of cells bearing vectors. SLN:DNA ratio (w/w) was 5:1. Error bars represent SD (n = 3). *p < 0.01 in transfection against to ARPE-19 cells. **p < 0.01 in uptake against to ARPE-19 cells. ***p < 0.05 in uptake against to ARPE-19 cells.

3.3 Cell uptake and intracellular distribution of Nile Red-labelled vectors

In order to evaluate the entry of the vectors into the cells SLNs were labelled with Nile Red, and they were bound to non-labelled plasmid. We compared the percentage of Nile Red positive HEK293 and ARPE-19 cells by flow cytometry. The lines in Figure 2 feature the results. In HEK293 cells levels were higher and did not vary along time; between 50% and 60% of the cells showed red fluorescence during the 72 h the experiment lasted. In ARPE-19 cells, during the first 4 h the percentage of Nile Red positive cells ranged from 10% to 20%, and at 72 h, cells containing nanoparticles increased to 55.8%, similar to the uptake level detected in HEK293 cells.

Figure 3 summarizes CLSM images captured from 2 to 72 h after the addition of vectors to the cells. After 2 h HEK293 cells (Figure 3A) showed vectors distributed

homogenously in all the cytoplasm. However, in ARPE-19 cells (Figure 3E) red fluorescence was mainly observed near the nucleus, in the region corresponding to the endoplasmic reticulum. At 4 h, in the two cell lines vectors were distributed around the nucleus. Twenty-four h after the addition of the vectors EGFP (green fluorescence) was only detected in HEK293 cultures (Figure 3C). Finally, at 72 h both cell lines showed EGFP and red fluorescence (Figure 3D and 3H). In ARPE-19 cells fluorescence of the vectors was very intense and appeared homogeneously distributed in the cytoplasm.

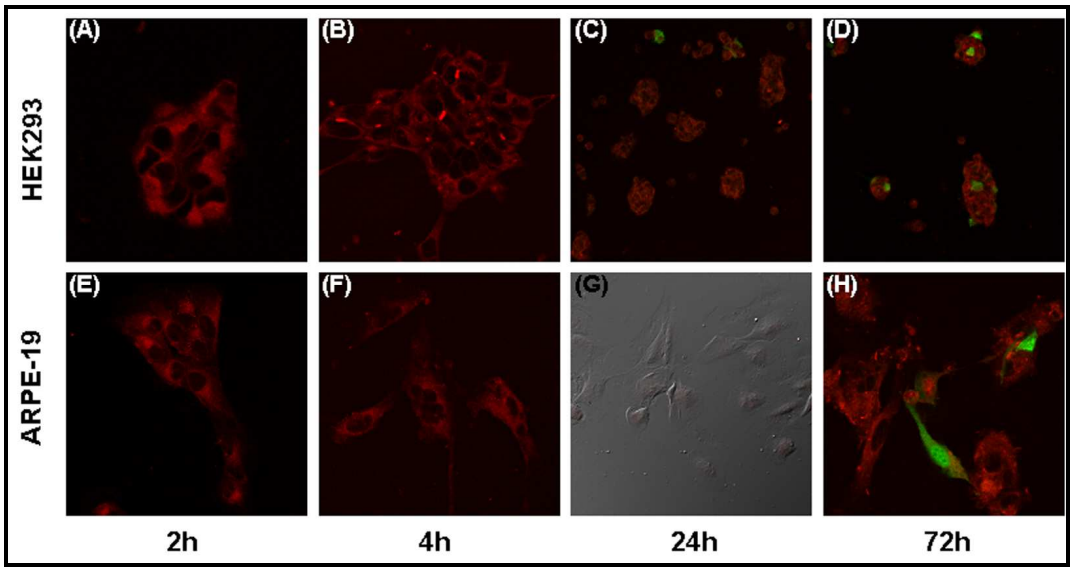

Figure 3. Confocal images of HEK293 cells (A)–(D) and ARPE-19 cells (E)–(H) at different times after the addition of SLN-DNA vectors. (A) and (E) 2 h; (B) and (F) 4h; (C) and (G) 24h; (D) and (H) 72 h. Image in panel G was captured with transmitted light in order to evidence the lack of fluorescent cells. Cells were treated with Nile Red-labelled SLN-DNA vectors (red) carrying the plasmid pCMS-EGFP, which encodes EGFP (green). SLN:DNA ratio (w/w) 5:1.

3.4 Co-localization assay

We also studied vectors entry mechanism in each cell line by CLSM. Co-localization studies of vectors with AlexaFluor488-labelled Transferrin and Cholera Toxin (Figure 4) indicated that SLN-DNA vectors showed co-localization with Cholera Toxin in HEK293

cells and with Transferrin in ARPE-19 cells. These findings indicate that in retinal cells SLN-DNA vectors mainly entered by clathrin-mediated endocytosis and in HEK293 they entered via caveolae.

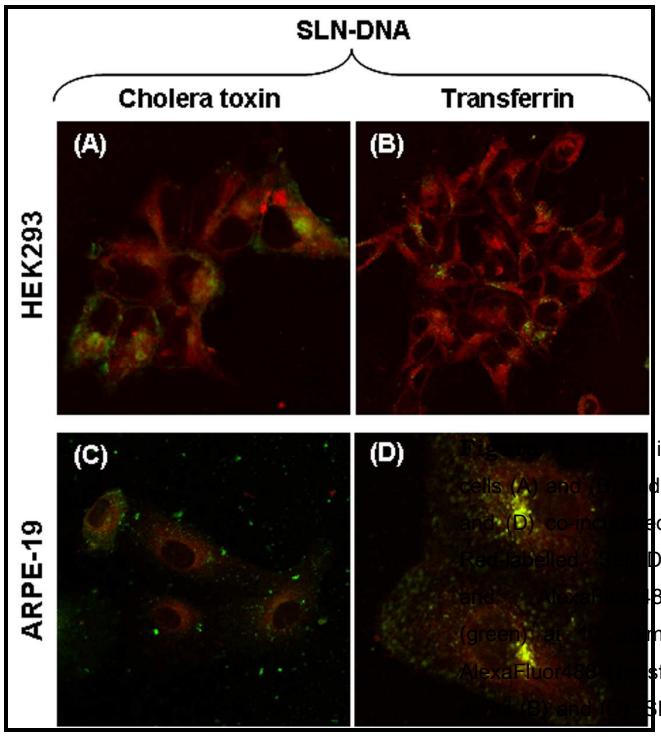

Fig. 5. [illegible] images of HEK293 cells (A) and (B) and ARPE-19 cells (C) and (D) co-incubated for 1 h with Nile Red-labelled SLN-DNA vectors (red) and [illegible]-Cholera toxin (green) at [illegible] ml (A) and (C) or AlexaFluor488-Transferrin (green) at 50 [illegible] (B) and (D). SLN:DNA ratio (w/w)

3.5 Intracellular distribution of EMA-labelled DNA in culture cells

In order to study the behaviour of DNA into the cells we employed the plasmid pCMS-EGFP labelled with ethidium monoazide (EMA) to elaborate SLN-DNA vectors. We also treated cells with Hoechst 33258 for the localization of the nucleus. Figure 5 represents images captured by fluorescence microscopy at 24 h after the addition of the vectors. At 72 h HEK293 cells hardly showed any red fluorescence (Figure 5B), while in ARPE-19 cells (Figure 5D) the presence of labelled DNA increased with respect to observations at 24 h.

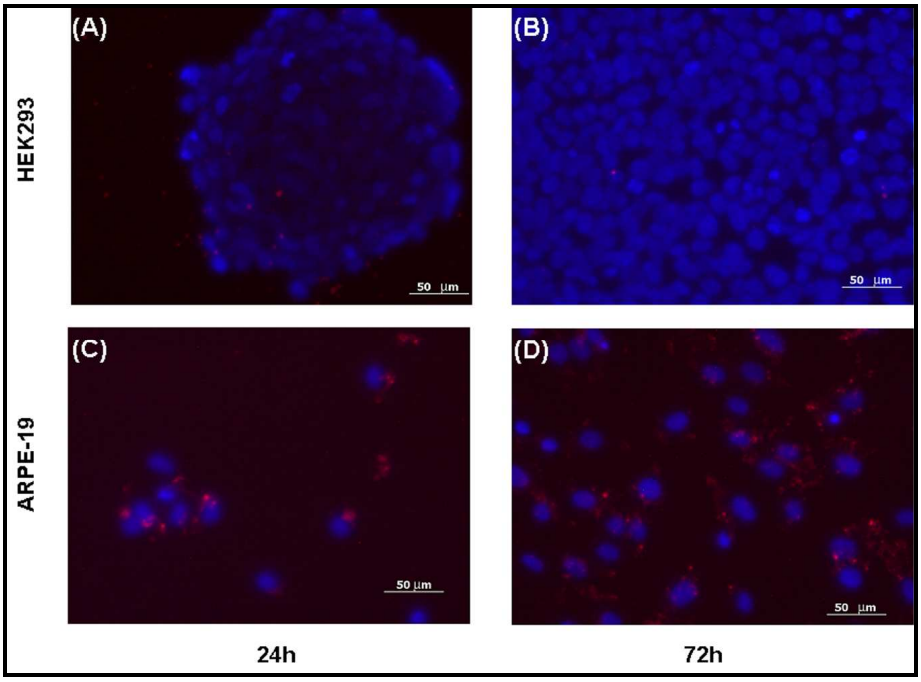

Figure 5. Fluorescence microscopy images of HEK293 cells (A) and (B) and ARPE-19 cells (C) and (D) at different times after the addition of SLN-DNA vectors. (A) and (C) 24 h; (B) and (D) 72 h. Cells were treated with Hoechst 33258 in order to detect the nucleus (blue) and vectors containing EMA-labelled pCMS-EGFP plasmid (red). SLN:DNA ratio (w/w) 5:1.

4. DISCUSSION

The transfection capacity of solid lipid nanoparticles (SLNs), composed by Precirol® ATO 5, DOTAP and Tween-80[23] has been evaluated in the human retinal pigment epithelial established cell line (ARPE-19). The flow cytometry study (bars in Figure 2) showed that SLNs are able to transfect ARPE-19 cells although transfection levels were lower than in HEK293 cells (2.5% vs. 14.9% EGFP positive cells at 72 h). In order to find the reason for the low transfection level in ARPE-19 cells, we performed a trafficking study of the SLNs in both cell lines.

Firstly, we studied the entry of Nile Red-labelled vectors by flow cytometry. In Figure 2 (lines) we can observe that at 0.5 h after the addition of the vectors, 50% of the HEK293 cells contained vectors, and this level was maintained until 72 h. In ARPE-19 cells, during the first 4 h positive cells ranged from 10% to 20%, and at 72 h, cells containing nanoparticles increased to 55.8%, a level similar to the uptake observed in HEK293 cells. Based on these results, a delay in transfection in ARPE-19 cells with respect to the HEK293 cells is expected. Transfection levels (Figure 2, bars) confirmed this delay. Green fluorescence was detected at 24 h in HEK293 cells, but in ARPE-19 cells, green fluorescence was not observed until 72 h.

Once the vector is inside the cells, the transport of DNA through the cytoplasm to the nucleus also plays an important role on transfection. The mobility of DNA molecules is difficult due to their large size and they can be degraded by cytoplasmic components. SLNs condense the DNA and reduce its size to facilitate the mobility and protection of the plasmid from components such as DNases[23].

The intracellular processing of the complex and its intracellular fate is affected by the pathway of entry into the cells. Endocytosis has been postulated as the main mechanism of entry for non-viral systems. Multiple mechanisms of endocytosis have been described, such as phagocytosis, pynocytosis, clathrin-mediated and caveolae-mediated. The relative contribution of each pathway in the lipoplex internalization has been poorly defined to date, although clathrin mediated endocytosis has been described as the main pathway for cationic lipid-based systems, and specifically, for lipoplexes composed by the

cationic lipid DOTAP and DNA. Rejman et al.[16, 28] concluded that the uptake occurs solely by the clathrin mediated mechanism.

Clathrin-mediated endocytosis leads to an intracellular pathway in which endosomes fuse with lysosomes degrading their content, whereas caveolae-mediated endocytosis avoids the lysosomal pathway and vector degradation. Hence, a timely release of the DNA from the endosomal compartment is essential in the clathrin pathway. Hoekstra et al.[29] have studied the endosomal escape of cationic lipid vectors and they describe that the non-lamellar phase changes of the lipoplexes, facilitated by intracellular lipids, allow DNA to dissociate from the vector and destabilize endosomal membranes.

Confocal images in Figure 3 feature the intracellular disposition of the SLN-DNA vectors in HEK293 (A)–(D) and ARPE-19 cells (E)–(H). In HEK293 cells (Figure 3A) red fluorescence was homogenously dispersed, indicating that vectors were taking up almost all the cytoplasm. However, in retinal cells (Figure 3E) vectors were mainly located in the same region that the rough endoplasmic reticulum and the Golgi apparatus, where lysosomes are produced. Thus, in ARPE-19 cells, the exposure of vectors to lysosomal digestive enzymes seems to be higher than in HEK293. Although SLNs are expected to entry by clathrin-mediated endocytosis as described for other lipid systems, differences observed in the intracellular localization between the two cell lines may be due to differences in the uptake mechanism: clathrin-mediated endocytosis in ARPE-19 cells vs. caveolae-mediated in HEK293 cells.

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28 Rejman J, Conese M, Hoekstra D. Gene transfer by means of lipo- and polyplexes: role of clathrin and caveolae-mediated endocytosis. J Liposome Res. 2006; 16: 237–247.

29 Hoekstra D, Rejman J, Wasungu L, Shi F, Zuhorn I. Gene delivery by cationic lipids: in and out of an endosome. Biochem Soc Trans. 2007; 35: 68–71.

In order to confirm the previous hypothesis, colocalization studies were carried out with AlexaFluor488-Cholera toxin and AlexaFluor488-Transferrin, which are markers for caveolae-mediated endocytosis and clathrin mediated endocytosis, respectively[30,31]. Results presented in Figure 4 revealed that SLN-DNA vectors colocalize with Cholera Toxin in HEK293 cells and mainly with Transferrin in ARPE-19 cells. These findings confirmed that SLN-DNA vectors mainly entered retinal cells by clathrin-mediated endocytosis and only via caveolae in HEK293. A higher clathrin-dependent entry and later internalization process in the ARPE-19 cell line compared to HEK293 cells may justify, in part, the lower transfection of the vectors in retinal cells.

Douglas et al.[32] studied the transfection of alginate-quitosan nanoparticles in different cell lines and concluded that clathrin-mediated internalization is required for efficient transfection: complexes that entered cells through caveolae-mediated processes were not trafficked to the endolysosomal pathway and, thus, were unable to escape from the vesicles and remained trapped and ineffective for transfection. However, our lipidic nanoparticles were able to transfect HEK293 cells in spite of their internalization into cells via caveolae. This indicates a successful escape from the caveosomes. It is known that the internalization process depends on the cell line but the composition of the formulation also conditions the endocytosis mechanism. Rejman et al.[27] and Kanatani et al.[33] also

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30 Pelkmans L, Helenius A. Endocytosis via caveolae. Traffic. 2002; 3: 311–320.

31 Pujals S, Fernandez-Carneado J, Ludevid D, Giralt E. D-SAP: a new, non cytotoxic and fully protease resistant cell-penetrating peptide. ChemMedChem. 2008; 3: 296–301.

32 Douglas KL, Piccirillo CA, Tabrizian M. Cell line-dependent internalization pathways and intracellular trafficking determine transfection efficiency of nanoparticle vectors. Eur J Pharm Biopharm. 2008; 68: 676–687.

33 Kanatani I, Ikai T, Okazaki A, Jo J, Yamamoto M, Imamura M, et al. Efficient gene transfer by pullulan-spermine occurs through both clathrin- and raft/caveolae-dependent mechanisms. J Control Release. 2006; 116: 75–82.

showed transfection via caveolea with polyplexes and lipofectamine, respectively. The knowledge of the endocytosis mechanisms should help us to design more efficient formulations. On the one hand, the entrance mechanism could be targeted to caveolae to prevent lysosomal degradation; on the other hand, the endosome escape can be favoured in the case of clathrin-mediated endocytosis. The use of additives as peptides[34,31] favours the cell uptake via caveolae and the incorporation of co-lipids as DOPE or cholesterol[35] helps endosome escape. Further similar research studies using other cell lines would ensure and reinforce the findings reported here.

Before entering into the nucleus, the DNA must be released from the complexes and this may be one of the most crucial steps for transfection. The capacity of the vector to condensate the DNA conditions its release profile. In a previous study[23] we showed that the DNA was able to be released from the SLN. The entry into the nucleus is in general quite difficult, as the nuclear membrane is a selective barrier to molecules bigger than 40 kDa, and plasmids surpass that size. There are two mechanisms those molecules can use to overcome that barrier: the disruption of the nuclear membrane during mitosis or the import through nuclear pore complex (NPC). This latter mechanism requires nuclear localization signals (NLS), which can be used to improve transfection by non-viral vectors[36]. Since our vectors do not have any NLS, plasmid DNA will enter the nucleus

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only during mitosis when the nuclear membrane transiently disappears. The division of HEK293 cells is faster than in ARPE-19 cells[37]; therefore, the entry of plasmid into the nucleus will be more difficult in ARPE-19 cells. The importance of division rate on the transfection process for cationic lipid-based systems has been already demonstrated[38, 39]. The slower division rate of ARPE-19 cells induces a delay in the entry of the DNA into their nucleus and the transduction will occur later. Therefore, the delay observed in the protein expression in ARPE-19 cells with respect to HEK293 cells (Figure 2) will be due not only to a delay in the entrance of the vector into the cell but also to a slower division rate.

In Figure 5 we can see the distribution of the DNA inside the cell by using vectors containing DNA labelled with ethidium monoazide (EMA), a red fluorescent DNA intercalating agent. In ARPE-19 cells, unlike in HEK293 cells, red fluorescence was higher at 72 h than at 24 h, and the DNA was close to the nucleus. As mentioned above, this is explained by a delay in the internalization uptake and also due to a slower cell division. These results made us think that in this cell line transfection will take a longer time than in HEK293. However, we tested transfection at day 7 (data not shown) and transfection decreased. This could be explained because the DNA stayed longer in the cytoplasm and was more exposed to degradation by different cytoplasmic agents such as DNAses. NLS can be incorporated into the DNA complexes in order to direct the plasmid

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37 Thomas P, Smart TG. HEK293 cell line: a vehicle for the expression of recombinant proteins. J Pharmacol Toxico Methods. 2005; 51: 187–200.

38 Mortimer I, Tam P, MacLachlan I, Graham RW, Saravolac EG, Joshi PB. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. Gene Ther. 1999; 6: 403–411.

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into the nucleus, shortening the stay of the plasmid in the cytoplasm and hence, decreasing DNA degradation. Subramanian et al.[40] reported an increase from 5% to 80% in transfection of non-dividing cells by the incorporation of NLS in their lipoplexes.

5. CONCLUSIONS

In conclusion, differences in internalization of the vectors into the two cell lines explain, in part, the difference in the gene expression. The lower transfection level obtained with the SLNs in ARPE-19 cells is due mainly to the cell uptake by a clathrin-mediated endocytosis that directs the solid lipid nanoparticles to lysosomes; moreover, the low division rate hampers the entrance of DNA into the nucleus. The study of the intracellular trafficking is a very useful tool for the designing of more efficient vectors taking into account the characteristics of the specific cell line to be transfected.

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A PROLINE-RICH PEPTIDE IMPROVES CELL TRANSFECTION OF SOLID LIPID NANOPARTICLE-BASED NON-VIRAL VECTORS

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ABSTRACT

The aim of this work was to improve the transfection efficacy of solid lipid nanoparticle(SLN)-based non-viral vectors into ARPE-19 cells through the addition of Sweet Arrow Peptide (SAP). First, we prepared SAP-DNA complexes at ratios of at least 50:1, and then incorporated them into the SLNs. All formulations were able to protect DNA, and the peptide favoured the most bioactive form (supercoiled) of open circular DNA turns. "*In vitro*" transfection studies of the vectors containing the pCMS-EGFP plasmid in HEK293 and ARPE-19 cell lines revealed that incorporation of SAP led to greater transfection in both cell lines, although via different mechanisms. The presence of SAP in the formulations did not affect the viability of HEK293 or ARPE-19 cells. In HEK293 cells, SAP enabled greater uptake of the vectors, and an SAP:DNA ratio of 50:1 was sufficient for enhancing transfection. In contrast, in ARPE-19 cells, SAP induced a change in the dominant entrance mechanism, from clathrin endocytosis to caveolae/raft-dependent endocytosis, thereby decreasing use of the lysosomal pathway and consequently, reducing vector degradation. The extent to which SAP uses one mechanism or the other largely depends on its concentration in the formulation.

Keywords: Solid lipid nanoparticles; cell penetrating peptide; transfection; intracellular trafficking; endocytic pathway.

1. INTRODUCTION

Gene therapy is a fledgling field of medicine with great potential for the treatment of inherited and acquired diseases. It encompasses a wide group of pharmaceuticals known as *gene delivery systems*, which are designed for the targeted delivery of DNA into cells. These systems include viral and non-viral vectors. Viral-based systems are the most effective, but their application is limited by the immunogenicity, oncogenicity and the small size of the DNA they can transport. Non-viral vectors, are safer, cheaper, more reproducible, and are not limited in the size of DNA they can transport; however, they suffer from low transfection efficacy. Non-viral vectors based on cationic lipids are being extensively studied[1,2,3,4,5,6]. Cationic lipids can be formulated in different ways, including as solid lipid nanoparticles (SLNs), which have shown transfection capacity "*in vitro*"[1,4,6].

The transfection process is dictated by factors such as the entry mechanism and the intracellular trafficking of the gene vectors employed. Trafficking of DNA to the nucleus involves steps which are common to all cell lines: entry into the cell, release into the cytoplasm, entry into the nucleus and transduction. However, the behaviour of a given

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nonviral system during these steps can vary with the cell line. Therefore, the study of intracellular trafficking—whereby the features of the specific cell line to be transfected are considered—is invaluable for improving upon existing vectors.

Our work is focused on the design of an effective DNA delivery system for the treatment of retinal diseases. Although gene therapy with viral vectors has proven effective for some retina related diseases[7,8,9], non-viral vectors are being extensively studied as an alternative due to their lower associated risks. Retinal Pigment Epithelial (RPE) cells are usually employed "*in vitro*"[10,11,12,13,14] for evaluation of new strategies in gene therapy for retinal diseases. In a previous work[15] we studied the intracellular

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trafficking of solid lipid nanoparticles in Human Retinal Pigment Epithelial (ARPE-19) and HEK293 cells. Differences in the uptake mechanism and subsequent internalization process partly justified the lower transfection levels observed for our vector in ARPE cells as compared to in HEK293 cells.

A novel approach to intracellular delivery of macromolecules is the use of peptides that can translocate through the cellular membrane and then, once inside the cell, unload their cargo to a targeted site. These are called cell penetrating peptides (CPPs)[16]. Among the most widely employed CPPs for DNA delivery are transcriptional activator protein (TAT)[17,18,19,20], Penetratin (pAntp)[21] and VP22[22,23], all of which have an infectious origin. In order to avoid the immunological risks associated with these peptides, alternatives such

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16 Gupta B, Levchenko TS, Torchilin VP. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. Adv Drug Deliv Rev. 2005; 57: 637-651.

17 Hashida H, Miyamoto M, Cho Y, Hida Y, Kato K, Kurokawa T, et al. Fusion of HIV-1 Tat protein transduction domain to polylysine as a new DNA delivery tool. Br J Cancer. 2004; 90: 1252-1258.

18 Hyndman L, Lemoine JL, Huang L, Porteous DJ, Boyd AC, Nan XS. HIV-1 Tat protein transduction domain peptide facilitates gene transfer in combination with cationic liposomes. J Control Release. 2004; 99: 435-444.

19 Ignatovich IA, Dizhe EB, Pavlotskaya AV, Akifiev BN, Burov SV, Orlov SV, et al. Complexes of plasmid DNA with basic domain 47-57 of the HIV-1 Tat protein are transferred to mammalian cells by endocytosis-mediated pathways. J Biol Chem. 2003; 278: 42625-42636.

20 Rudolph C, Plank C, Lausier J, Schillinger U, Müller RH, Rosenecker J. Oligomers of the arginine-rich motif of the HIV-1 TAT protein are capable of transferring plasmid DNA into cells. J Biol Chem. 2003; 278: 11411-11418.

21 Christiaens B, Dubruel P, Grooten J, GoethalsM, Vandekerckhove J, Schacht E, et al. Enhancement of polymethacrylate-mediated gene delivery by Penetratin. Eur J Pharm Sci. 2005; 24: 525-537.

22 Suzuki K, Murtuza B, Brand NJ, Varela-Carver A, Fukushima S, Yacoub MH. Enhanced effect of myocardial gene transfection by VP22-mediated intercellular protein transport. J Mol Cell Cardiol. 2004; 36: 603-606.

23 Zender L, Kock R, Eckhard M, Frericks B, Gosling T, Gebhardt T, et al. Gene therapy by intrahepatic and intratumoral trafficking of p53-VP22 induces regression of liver tumors. Gastroenterology. 2002; 123: 608-618.

as polyarginines[24] or proline-rich peptides[25] have been developed. Among proline-rich peptides, the amphipathic Sweet Arrow Peptide (SAP), whose sequence is $(VRLPPP)_3$ (made with three repetitive VRLPPP units; V = Val, R = Arg, L = Leu, and P = Pro), has demonstrated good translocation properties and is non-cytotoxic[26].

The aim of this work was to improve the transfection of SLN-based non-viral vectors into ARPE-19 and HEK293 cells by adding SAP to the formulation. We rationalized that combination of SLNs with SAP could facilitate vector entry and subsequent intracellular trafficking in ARPE-19 cells. We evaluated the transfection capacity of the SLN-SAP vectors, and then correlated it to their cellular uptake and intracellular behaviour in ARPE-19 and HEK293 cells.

2. MATERIALS AND METHODS

2.1 Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTAP), deoxyribonuclease I (DNase I), lauryl sulphate sodium (SDS), antibiotic/antimycotic, Nile Red, Filipin III and chlorpromazine were purchased from Sigma-Aldrich (Madrid, Spain). Tween-80 was provided by Vencaser (Bilbao, Spain), and dichloromethane, by Panreac (Barcelona, Spain).

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24 Belting M, Sandgren S, Wittrup A. Nuclear delivery of macromolecules: barriers and carriers. Adv Drug Deliv Rev. 2005; 57: 505-527.

25 Fernández-Carneado J, Kogan MJ, Pujals S, Giralt E. Amphipathic peptides and drug delivery. Biopolymers. 2004; 76: 196-203.

26 Fernández-Carneado J, Kogan MJ, Castel S, Giralt E. Potential peptide carriers: Amphipathic proline-rich peptides derived from the n-terminal domain of gamma-zein. Angew Chem Int Edit. 2004; 43: 1811-1814.

The plasmid pCMS-EGFP, which encodes the enhanced green fluorescent protein (EGFP), was purchased from BD Biosciences Clontech (Palo Alto, California, US) and amplified by Dro Biosystems S.L. (San Sebastián, Spain).

The gel electrophoresis materials were acquired from Bio-Rad (Madrid, Spain). AlexaFluor488-Cholera toxin and AlexaFluor488-Transferrin were provided by Molecular Probes (Oregon, US)

Cell culture reagents were purchased from LGC Promochem (Barcelona, Spain). Antibiotic Normocin™ was acquired from InvivoGen (San Diego, California, US).

BD Viaprobe kit was provided by BD Biosciences (Belgium). Fluoromount G was purchased from SouthernBiotech (Coultek, Spain).

2.2 Production of solid lipid nanoparticles (SLNs)

The SLNs were produced by a solvent emulsification-evaporation technique[27]. Precirol® ATO 5 was dissolved in dichloromethane (5% w/v), and then emulsified in an aqueous phase containing DOTAP (0.4% w/v) and Tween 80 (0.1% w/v). The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) for 30 s at 50 W. The organic solvent was then removed by evaporation using a magnetic agitator for 45 min, followed by vacuum conditions for 15 min. An SLN suspension was formed upon precipitation of the Precirol® ATO 5 in the aqueous medium. Finally, the SLNs were washed by centrifugation (3,000 rpm, 20 min, x 3) using Millipore (Madrid, Spain) Amicon® Ultra centrifugal filters (100,000 MWCO).

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27 Mehnert W, Mader K. Solid lipid nanoparticles - Production, characterization and applications. Adv Drug Deliv Rev. 2001; 47: 165-196.

2.3 Synthesis of Sweet Arrow Peptide (SAP)

SAP was synthesized on 2-chlorotrityl chloride resin using the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) strategy and TBTU or PyBOP as coupling reagent. The peptide was cleaved using a cleavage mixture of 95% TFA, 2.5% TIS and 2.5% water. It was purified by semipreparative RP-HPLC, and then characterized by analytical RP-HPLC and MALDI-TOF. For further details, see Ref **26**.

2.4 Studying the binding of SAP to DNA

pCMS-EGFP plasmid DNA was added to an aqueous solution of SAP at SAP:DNA ratios (w/w) of 1:1, 2:1, 5:1, 10:1, 50:1, 100:1 and 200:1. The resulting complexes were diluted in Milli-Q™ water up to a final concentration of 0.1 μg DNA/μL, and then subjected to electrophoresis on a 0.8% agarose gel (containing 1% ethidium bromide for visualization) for 30 min at 120 V. The bands were observed with a Vilber-Lourmat TFX-20M transilluminator. Images were captured using a Bio-Rad DigiDoc digital camera.

2.5 Preparation of vectors

SLN-DNA vectors were obtained by mixing the pCMS-EGFP plasmid with an aqueous suspension of SLNs. The SLN:DNA ratio, expressed as the ratio of DOTAP:DNA (w/w), was fixed at 5:1.

SAP-DNA-SLN vectors were prepared by first forming SAP-DNA complexes at the desired ratios, and then incorporating the SLNs under agitation for 30 min. We first bound SAP to DNA resulting in a SAP-DNA complex, which possess negative charge. Then, SAP-DNA complexes were put in contact with a suspension of previously prepared SLNs, and electrostatic interactions between SAP-DNA complexes and SLNs led to the

formation of SAP-DNA-SLNs vectors. These vectors have SAP-DNA complexes adsorbed on the nanoparticle surface.

2.6 Size and zeta potential measurements

Sizes of SLNs, SLN-DNA and SAP-SLN-DNA vectors were determined by photon correlation spectroscopy (PCS). Zeta potentials were measured by laser Doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in 0.1 mM NaCl (aq.).

2.7 DNase I protection study

DNase I was added to SLN-DNA and SAP-DNA-SLN vectors to a final concentration of 1 U DNase I/2.5 μg DNA, and the mixtures were then incubated at 37 ºC for 30 min. Afterwards, 2% SDS solution was added to the samples to a final concentration of 1% to release the DNA from the SLNs. The samples were then analyzed by electrophoresis on agarose gel (described above), and the integrity of the DNA in each sample was compared with a control of untreated DNA.

2.8 Cell culture and transfection protocol

"*In vitro*" assays were performed with two different cell lines: Human Embryonic Kidney (HEK293) and Human Retinal Pigment Epithelial (ARPE-19), obtained from the American Type Culture Collection (ATCC).

HEK293 cells were maintained in Eagle´s Minimal Essential Medium with Earle´s BSS and 2 mM L-glutamine (EMEM) supplemented with 10% heat-inactivated horse serum and 1% antibiotic/antimycotic. Cells were incubated at 37 ºC under 5% CO_2 atmosphere

and subcultured every 2 to 3 days using trypsin-EDTA. For transfection, HEK293 cells were seeded on 24 well plates at a density of 150,000 cells per well and allowed to adhere overnight.

ARPE-19 cells were maintained in Dulbecco's Modified Eagle's Medium-Han's Nutrient Mixture F-12 (1:1) medium (D-MEM/F-12) supplemented with 10% heat-inactivated fetal calf serum and 1% Normocin™ antibiotic solution. Cells were incubated at 37 ºC under 5% CO_2 atmosphere and subcultured every 2 to 3 days using trypsin-EDTA. For transfection, ARPE-19 cells were seeded on 12 well plates at a density of 30,000 cells per well and allowed to adhere overnight.

The different formulations were diluted in HBS and added to the cell cultures. In all cases, 2.5 μg of DNA were added. The cells were incubated with the vectors at 37 ºC, and after 4 h, the medium containing the complexes in the wells was refreshed with 1 mL of complete medium. The cells were then allowed to grow for another 72 h.

As control, an SLN-DNA vector whose transfection ability was evaluated in previous works[1,15] was also assayed.

2.9 Flow cytometry-mediated analysis of transfection efficacy and cell viability

At the end of the incubation period, the cells were washed once with 300 μL of PBS, and then detached with 300 μL of 0.05% trypsin-EDTA. The cells were then centrifuged at 1500g, and the supernatant was discarded. The cells were then resuspended with PBS and directly introduced into a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, California, US). For each sample, 10,000 events were collected.

Transfection efficacy was quantified by measuring the fluorescence of EGFP at 525 nm (FL1). For cell viability measurements, the BD Via-Probe kit was employed. This reagent was used to exclude dead cells from the analysis. The reagent (5 μL) was added to each sample, and after 10 min of incubation, the fluorescence corresponding to dead cells was measured at 650 nm (FL3). Dead cells were excluded from the analysis.

2.10 Cellular uptake of non-viral vectors

Entry of the vectors into the cells was studied quantitatively by flow cytometry and qualitatively by confocal laser scanning microscopy (CLSM), for which SLNs were labelled with the fluorescent dye Nile Red (λ = 590 nm) according to a previously reported[28] method. SLNs were prepared by the emulsification-evaporation technique described above, in which the Nile Red was incorporated into the dichloromethane.

At different time points cells were washed three times with PBS and detached from plates. Cells incorporating either SLN-DNA or SAP-DNA-SLN vectors were quantified by flow cytometry at 650 nm (FL3). For each sample, 10,000 events were collected.

For CLSM study, we first confirmed that the paraformaldehyde did not interact with the fluorescence of Nile Red or EGFP. The cells were seeded in coverslips containing plates and treated with either SLN-DNA or SAP-DNA-SLN vectors. At different incubation times, the medium was removed, and the cells were washed with PBS and fixed with 4% paraformaldehyde. Preparations were mounted on Fluoromount G. After air-drying, images were acquired with an Olympus Fluoview FV500 confocal microscope using

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28 Borgia SL, Regehly M, Sivaramakrishnan R, Mehnert W, Korting HC, Danker K, et al. Lipid nanoparticles for skin penetration enhancement-correlation to drug localization within the particle matrix as determined by fluorescence and parelectric spectroscopy. J Control Release. 2005; 110: 151-163.

sequential acquisition to avoid overlapping of fluorescent emission spectra. CLSM images were captured in the General Service of Analytical Microscopy and High Resolution in Biomedicine of the University of the Basque Country (UPV-EHU).

2.11 Mechanism of internalization

The endocytic processes involved in the internalization of the non-viral systems were analyzed qualitatively by co-localization studies with AlexaFluor488-Cholera toxin and AlexaFluor488-Transferrin, and quantitatively by using the endocytosis inhibitors filipin III and chlorpromazine.

For co-localization studies, cells were seeded in coverslips and co-incubated for 1 h with Nile Red labelled vectors (SLN-DNA or SAP-DNA-SLN) and either AlexaFluor488-Cholera toxin (10 μg/mL) or AlexaFluor488-Transferrin (50μg/mL), which are markers for caveolae/raft-mediated endocytosis and clathrin-mediated endocytosis, respectively. Samples were later analyzed by CLSM. Cell fixation and image acquisition were performed as described in Section 2.10.

Uptake inhibition induced by filipin III or chlorpromazine was checked by measuring cell uptake and cell viability by flow cytometry. ARPE-19 cells were seeded on 12 well plates at a density of 30,000 cells. On the fourth day post-seeding the cells were equilibrated in 500 μL of serum-free medium at 37ºC, followed by incubation for 30 min with the inhibitors: filipin III (1 μg/mL) or chlorpromazine (20 μg/mL). Then, Nile Red labelled SLN-DNA or SAPDNA-SLN vectors were added to the cell cultures for further 60 min or 90 min (when the inhibitor filipin III or chlorpromazine were used, respectively). Then, cells incorporating either SLN-DNA or SAP-DNA-SLN vectors were quantified by flow cytometry as it is described in Section 2.10.

2.12 Statistical analysis

Results are reported as mean values (SD = standard deviation). Statistical analysis was performed with SPSS 14.0 (SPSS®, Chicago, IL, USA). Normal distribution of samples was assessed by the Shapiro–Wilk test, and homogeneity of variance, by the Levene test. The different formulations were compared with the Student´s t-test, whereby differences were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

Attaining satisfactory intracellular delivery is crucial for drug targets inside the cell (e.g. DNA). An alternative strategy is the use of peptide sequences that can translocate the cytoplasmic membrane, called cell penetrating peptides (CPPs). Many CPPs contain positively charged amino acids and are hydrophobic; this is frequently due to an amphipathic sequence[29]. Sweet Arrow Peptide (SAP) crosses cell membranes, and it is advantageous over other carrier peptides owing to its non-viral origin, amphipathicity, aqueous solubility, and non-cytotoxicity, even at high concentrations[26].

The addition of peptides to gene delivery systems is a known method for lipofection enhancement[29,30]. The incorporation of SAP into our formulations facilitates entry of the vectors into the cells and subsequent intracellular trafficking, thereby increasing transfection efficacy.

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29 Khalil IA, Kogure K, Futaki S, Harashima H. Octaarginine-modified liposomes: Enhanced cellular uptake and controlled intracellular trafficking. Int J Pharm. 2008; 354: 39-48.

30 Rea JC, Barron AE, Shea LD. Peptide-mediated lipofection is governed by lipoplex physical properties and the density of surface-displayed amines. J Pharm Sci. 2008; 97: 4794-4806.

3.1 Binding of SAP to DNA

Positively charged amino acids (e.g. arginine) and hydrophobic interactions (e.g. valine and leucine) enable SAP to bind DNA. DNA condensation greatly affects the transfection capacity of SLNs, as it influences the delivery of DNA from nanoparticles, protection of genes from external agents, and DNA topology. We previously demonstrated that SLNs condense DNA[1], which facilitates mobility of the plasmid, as well as protection of it from components such as DNases.

For the binding assay, we combined peptide and plasmid at different SAP:DNA ratios, and then subjected the resulting complexes to electrophoresis on an agarose gel (Figure 1). The gel illustrates that the capacity of SAP to condense DNA depends on the SAP:DNA ratio. Lane 1 corresponds to free DNA. In lanes 2-5 (SAP:DNA ratios from 1:1 to 10:1), the intensity of the bands indicates that most DNA was free, whereas in lanes 6 and 7 (SAP:DNA ratios 50:1 and 100:1, respectively), the bands were less intense, and most DNA was retained at the point where the complexes had been placed. In lane 8, (SAP to DNA ratio 200:1) DNA is not totally retained in the initial point, but the release took place later, as DNA runs less distance. Therefore, a ratio of at least 50:1 is needed to obtain high DNA binding although DNA migration was not completely inhibited at any of the ratios assayed. Based on these results, we chose the ratios 50:1, 100:1 and 200:1 to prepare the SAP-DNA-SLN vectors.

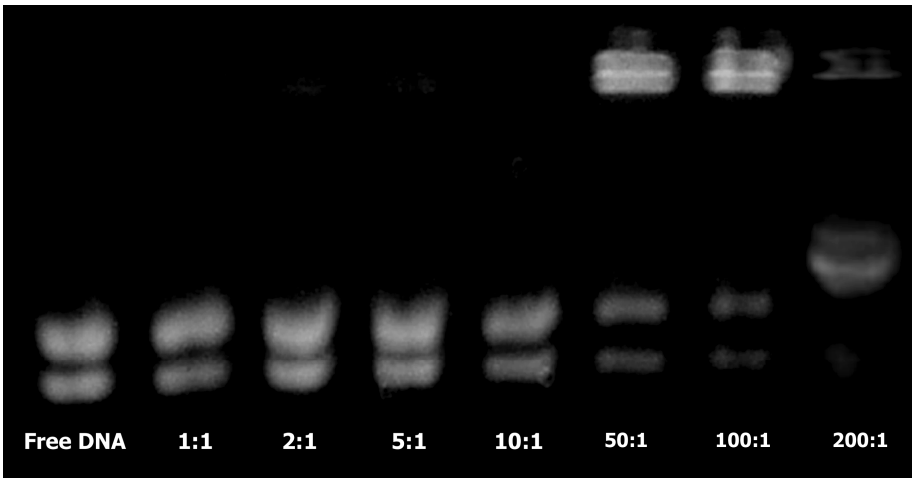

Figure 1. Binding efficiency of SAP to DNA at different ratios (w/w), as studied by agarose gel electrophoresis.

3.2 Characterization

3.2.1 Particle size and zeta potential of the formulations

Peptides can potentiate lipid-mediated gene delivery by modifying lipoplex properties. First, we studied the influence of SAP on the properties of the formulations, namely, surface charge and particle size—both of which influence the mechanism of cell internalization[31]. Table 1 lists the particle size and zeta potential of each formulation. SAP-DNA vectors showed negative zeta potential (from -16 to -19 mV), but size resulted too small to be measured by PCS. When SAP-DNA complexes were bound to SLNs, SAP did not induce any significant change in surface charge ($p > 0.05$), but it did affect particle size of SLN. At an SAP:DNA ratio of 50:1, the size of the formulation was reduced to only 210 nm, as compared to 299 nm in the control formulation ($p < 0.01$). At a

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31 Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin-and caveolae-mediated endocytosis. Biochem J. 2004; 377: 159-169.

ratio of 100:1, the reduction was lower (down to 234 nm) ($p < 0.05$), and at 200:1, no significant differences in size as compared to the control were found. This trend could be due to a balance between the ability of the peptide to precondense DNA, which would imply a reduction in size, while demanding greater space itself, which would cause an increase in size.

Table 1. Size and zeta potential of non-viral vectors prepared at a SLN:DNA ratio (w/w) of 5:1, and different SAP:DNA ratios; the ratio SLN:DNA is expressed a w/w ratio of DOTAP:DNA.

Formulation	Size (nm)	Zeta potential (mV)
SLN-DNA	299 (49)	+ 33 (6)
SAP-DNA-SLN 50:1:5	210 (40) *	+ 30 (12)
SAP-DNA-SLN 100:1:5	234 (33) **	+ 30 (5)
SAP-DNA-SLN 200:1:5	254 (82)	+ 28 (4)

Mean values (SD = Standard deviation), for n=3

*$p < 0.01$ respect to SLN-DNA formulation.

**$p < 0.05$ respect to SLN-DNA formulation.

3.2.2 "*In vitro*" resistance against DNase I

As mentioned above, an important advantage of non-viral systems in gene therapy is their capacity to protect DNA from components of the medium, above all from digestion by DNases. We studied the protection capacity of the SLN-DNA and SAP-DNA-SLN vectors, by analyzing the integrity of DNA in agarose gel electrophoresis after treatment with DNase I. Gel electrophoresis (Figure 2) shows that addition of SAP to the

formulations did not decrease the DNA protection capacity of the basic formulation SLN-DNA. Lane 1 corresponds to nontreated free DNA, and lane 2, to DNase I-treated free DNA. The absence of a band in this lane indicates that DNA was totally digested by the enzyme. Lane 3 corresponds to SLN-DNA vectors, and lanes 4 to 6 correspond to SAP-DNA-SLN formulations (SAP:DNA ratios 50:1, 100:1, 200:1, respectively) treated with DNase I. The bands in these lanes reveal that all formulations were able to protect DNA from nuclease action.

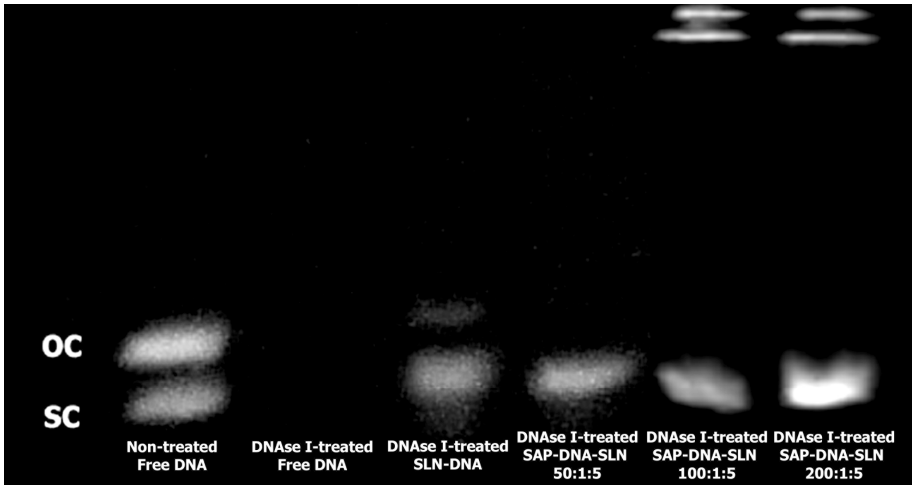

Figure 2. Protection of DNA from DNase I digestion by non-viral vectors, as visualized by agarose gel electrophoresis.

We also evaluated DNA topology. Non-treated free DNA (lane 1) and DNA from SLN-DNA complexes treated with DNAse I (lane 3) presented two bands. The lower band corresponds to supercoiled (SC) DNA, which has been reported in the literature to be the most bioactive form[32,33], and the upper band, to open circular (OC) DNA. However in

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32 Middaugh CR, Evans RK, Montgomery DL, Casimiro DR. Analysis of plasmid DNA from a pharmaceutical perspective. J Pharm Sci. 1998; 87: 130-146.

lanes 4 to 6, which corresponds to SAP-DNA-SLN vectors, only the SC band appeared, and it was more intense than in the case of the vectors lacking SAP (lane 3). This is related to the DNase I mechanism of action, which converts SC-DNA into OC-DNA by cutting one of the DNA double strands, and then breaking down the OC to L-DNA (linear DNA)[34], which appears between the other two bands. The absence of OC and L bands in the formulations containing SAP indicates that the peptide maintained the protective capacity of the control formulation and favours the conversion of OC to SC.

3.3 Transfection and cell viability studies in HEK293 and ARPE-19 cells

Once the vectors were characterized we studied transfection and viability in HEK293 and ARPE-19 cells. Prior to this evaluation we showed that SAP-DNA complexes were not able to transfect cells, which is probably a result of their negative charge, which stems from the low capacity of SAP to condensate DNA. Positively charged amino acids not only enable SAP to bind DNA, but are also important for electrostatic interactions with the anionic groups of the cell surface. Harush-Frenkel et al.[35] studied the endocytosis of nanoparticles carrying either a negative or positive surface charge, and concluded that the charge significantly affected internalization levels: the negatively charged nanoparticles were endocytosized to a lesser extent.

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33 Remaut K, Sanders NN, Fayazpour F, Demeester J, De Smedt SC. Influence of plasmid DNA topology on the transfection properties of dotap/dope lipoplexes. J Control Release. 2006; 115: 335-343.

34 Sanders NN, De Smedt SC, Demeester J. In: McGrath BM and Walsh G. (Eds.). Therapeutic Enzymes. 2006; Taylor & Fracis Group, Boca Raton, pp. 97-116.

35 Harush-Frenkel O, Debotton N, Benita S, Altschuler Y. Targeting of nanoparticles to the clathrin-mediated endocytic pathway. Biochem Biophys Res Commun. 2007; 353: 26-32.

The incorporation of SAP-DNA complexes into SLN provided us with a condensation more suited to our goal, as it enables us to bind an adequate amount of DNA while maintaining a positive final charge. We prepared SAP-DNA-SLN vectors, increasing SAP:DNA ratio in order to evaluate the influence of the amount of peptide on cell transfection and viability. Rea et al.[30] showed that the order of addition of the peptides and lipids was critical for enhancing lipofection: adding peptides prior to the lipids did not affect their presentation on lipoplex surfaces, however, adding them after leads to the exclusion of the small peptides from the lipoplexes. We first incorporated SAP into the SLN-DNA complex. At this point, no transfection was detected (data not shown), which is consistent with Rea´s work. The final formulations were then prepared and assayed by first precondensing the DNA with the SAP at the desired ratio, and then adding the resulting complexes to the SLN.

As observed in Figures 3A and 3C, transfection levels in HEK293 cell cultures were significantly higher ($p < 0.01$) than in ARPE-19 cells for all vectors assayed. We have previously reported[15] differences in the transfection capacity of the control formulation (SLN-DNA) between the two cell lines, which we attributed to the difference in the internalization process used in each line. Upon incorporation of SAP into the vectors, the transfection capacity remains higher in HEK293 cells.

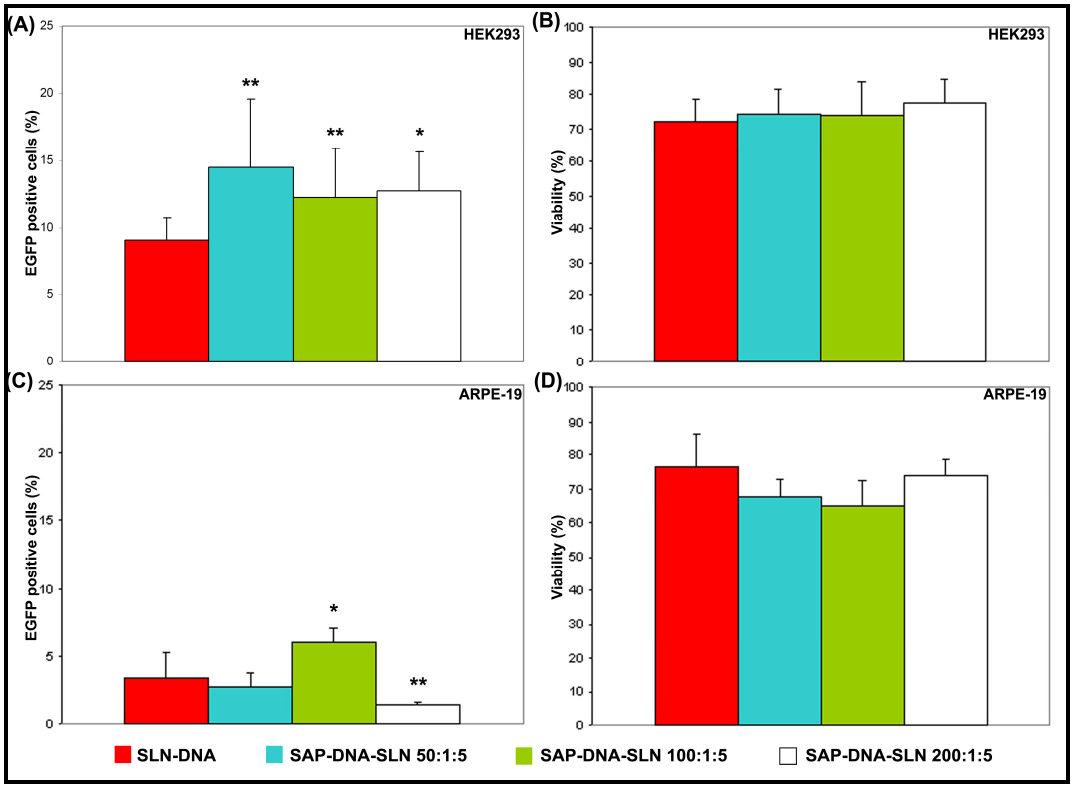

Figure 3. Transfection and cell viability for each formulation, assayed in HEK293 cells (A and B) and ARPE-19 cells (C and D); the SLN:DNA ratio (w/w) was 5:1 in all cases, and the SAP:DNA ratio varied from 50:1 to 200:1. * p < 0.01 respect to SLN-DNA formulation; ** p <0.05 respect to SLN-DNA formulation; Error bars represent SD (n=3).

We do not believe that the differences in transfection are due to the surface charge or size of the vectors, since there were no changes in surface charge, and reduction in particle size is minor (from 299 nm to 210 nm). Incorporation of SAP significantly increased the number of cells expressing enhanced green fluorescent protein (EGFP), and the effect on transfection efficacy depended on the cell line. The percentage of EGFP positive HEK293 cells increased from 9.1% in the control formulation to 14.5%, 12.3% and 12.8% for vectors prepared at SAP:DNA ratios 50:1, 100:1 and 200:1, respectively (Figure 3A). No differences were detected in cell transfection among the three

formulations containing SAP. In ARPE-19 cells, the percentage of EGFP positive cells only increased at an SAP:DNA ratio of 100:1, (Figure 3C), from 3.5 % to 6.0% ($p < 0.01$). In retinal cells no significant differences in the percentage of transfected cells were detected among SLN-DNA vectors or SAP-DNA-SLN vectors prepared at an SAP:DNA ratio of 50:1 ($p > 0.05$); this amount of SAP seems to be not enough to have influence on the transfection of these cells. Furthermore, the transfection efficacy decreased when the SAP:DNA ratio was 200:1. As we explained above, this reduction does not seem to be related to size or superficial charge of the vectors. ARPE-19 cells present a low division rate which hampers the entry of plasmids into the nucleus. In our opinion, a high amount of the peptide bound to DNA could difficult the plasmid to reach the nuclear machinery. The presence of SAP did not affect the viability of either HEK293 or ARPE-19 cells (Figures 3B and 3D, respectively).

3.4 Cellular uptake of non-viral vectors

Being that SAP is a CPP that can translocate the cytoplasmic membrane, we studied how it affects cell uptake of the vectors. We labelled vectors with the fluorophore Nile Red, and then used flow cytometry to compare the percentage of Nile Red positive cells in HEK293 and ARPE-19 cultures treated with either the control SLN-DNA formulation or SAP-DNA-SLN (SAP:DNA ratio of 100:1). As previously reported[15], the vectors entered into HEK293 cells (Figure 4A) more quickly than into ARPE-19 cells (Figure 4B). HEK293 cultures treated with the SAP-DNA-SLN had a higher percentage of Nile Red positive cells (Figure 4A) than did those treated with the control formulation, which is consistent with higher transfection levels for this cell line ($p < 0.05$). However, although transfection in ARPE-19 cultures with the SAP-DNA-SLN was almost double that with the control formulation (6.0% and 3.5%, respectively), the entry into cells (Figure 4B) was not

significantly different. We therefore concluded that in ARPE-19 cells, SAP increases cell transfection through a different mechanism than in HEK293.

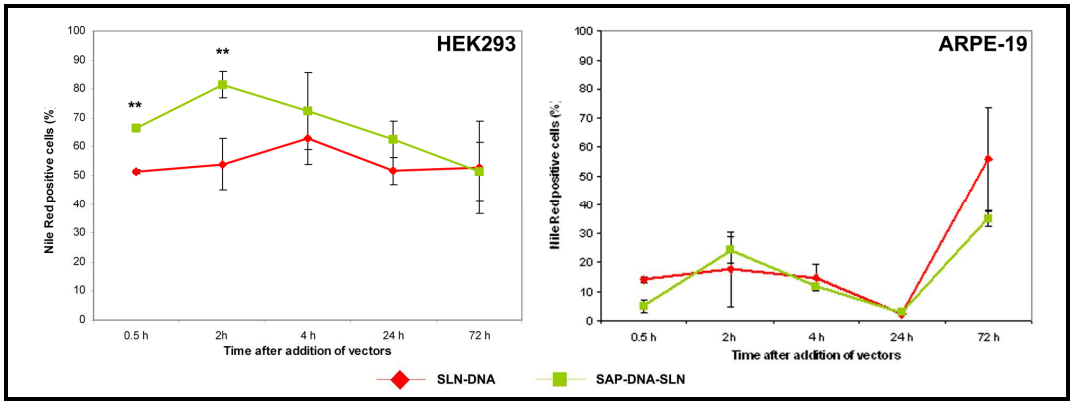

Figure 4. Uptake of non-viral vectors by HEK293 cells (A) and ARPE-19 (B) cells, represented as the percentage of cells containing Nile Red labelled vectors at different times after the addition of vectors. Cells were treated with either SLN-DNA (SLN:DNA ratio [w/w] of 5:1) or SAP-DNA-SLN vector (SAP:DNA ratio [w/w] of 100:1); ** $p < 0.05$ respect to SLN-DNA formulation; Error bars represent SD (n=3).

We then studied the behaviour of the vectors in the cytoplasm using CLSM. Figure 5 shows the distribution of the vectors in both cell lines over time. Confocal images of HEK293 cells (Figures 5A to 5G) show that the location of the control and SAP formulations was similar in the time period evaluated (from 2 to 72 h). However, in retinal cells, the formulations differed in location: at 2 h, the control vectors (Figure 5L) showed a scattered pattern in the endoplasmic reticulum and/or Golgi apparatus, whereas the SAP vectors (Figure 5I) were located around the nucleus. These differences in subcellular localization are probably due to differences in the mechanism of entry.

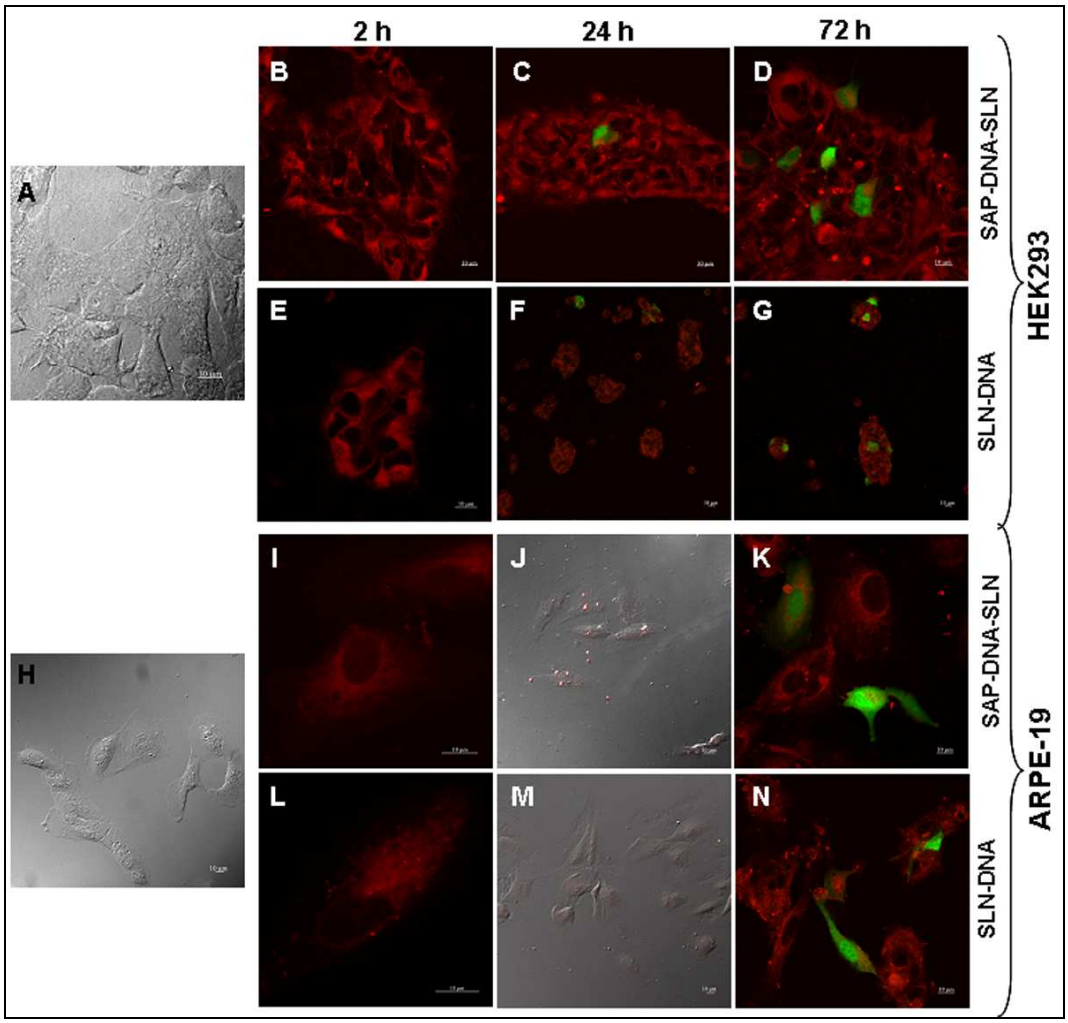

Figure 5. CLSM images of HEK293 cells (A to G) and ARPE-19 cells (H to N) at different times after the addition of vectors; B, E, I, L: 2 h; C, F, J, M: 24 h; D, G, K, N: 72 h; Images in panels A and H are bright fields of the HEK293 and ARPE-19 cells respectively. Images in panels J and M were captured with transmitted light in order to show the lack of fluorescent cells. Cells were treated with SAP-DNA-SLN vectors (B-D, I-K) or SLN-DNA vectors (E-G, L-N) at an SLN:DNA ratio (w/w) of 5:1 and an SAP:DNA ratio (w/w) of 100:1. Nile Red-labelled vectors in red, EGFP in green.

3.5 Mechanism of internalization

We also studied the mechanism of entry of SAP-vectors in both cell lines using CLSM. Endocytosis has been postulated as the main entry mechanism for non-viral systems.

Various mechanisms of endocytosis have been described to date: phagocytosis, pinocytosis, clathrin-mediated and caveolae/raft-mediated. Clathrin-mediated endocytosis leads to an intracellular pathway in which endosomes fuse with lysosomes which degrade their content, whereas caveolae/raft-mediated endocytosis avoids the lysosomal pathway and its consequent vector degradation. Previous studies have shown that in HeLa cells, SAP is internalized by caveolae/raft-dependent endocytosis by co-localization studies with cholera toxin[36,37]. It has been reported that cholera toxin is internalized by lipid rafts (complex, membrane-bound microdomains that contain high levels of cholesterol and sphingolipids), as well as by caveolae, which differ from lipid rafts only in that they contain caveolin-1, which stabilizes these domains in the plasma membrane[38,39,40]. These two carriers remain the subject of debate: some authors consider caveolae-mediated endocytosis to be a subclass of lipid-raft mediated endocytosis[41,42], whereas others have suggested using the same nomenclature for both mechanisms (*caveolae/raft-dependent*

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36 Foerg C, Ziegler U, Fernandez-Carneado J, Giralt E, Rennert R, Beck-Sickinger AG, et al. Decoding the entry of two novel cell-penetrating peptides in HeLa cells: Lipid raft-mediated endocytosis and endosomal escape. Biochemistry. 2005; 44: 72-81.

37 Pujals S, Fernandez-Carneado J, Ludevid D, Giralt E. D-SAP: A new, non-cytotoxic and fully protease resistant cell-penetrating peptide. ChemMedChem. 2008; 3: 296-301.

38 Nichols BJ, Kenworthy AK, Polishchuk RS, Lodge R, Roberts TH, Hirschberg K, et al. Rapid cycling of lipid raft markers between the cell surface and Golgi complex. J Cell Biol. 2001; 153: 529-541.

39 Orlandi PA, Fishman PH. Filipin-dependent inhibition of cholera toxin: Evidence for toxin internalization and activation through caveolae-like domains. J Cell Biol. 1998; 141: 905-915.

40 Puri V, Watanabe R, Singh RD, Dominguez M, Brown JC, Wheatley CL, et al. Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. J Cell Biol. 2001; 154: 535-547.

41 Anderson RGW. The caveolae membrane system. Annu Rev Biochem. 1998; 67: 199-225.

42 Kurzchalia TV, Parton RG. Membrane microdomains and caveolae. Curr Opin Cell Biol. 1999; 11: 424-431.

endocytosis)[43]; consequently, we use this nomenclature when co-localization with cholera toxin was observed.

We studied co-localization of the vectors using AlexaFluor488–labelled Transferrin and cholera toxin, which are markers for clathrin endocytosis and caveolae/raft-mediated endocytosis, respectively. We observed that in HEK293 cells all formulations enter by caveolae/raft-dependent endocytosis (Figures 6A to 6D), whereas in ARPE-19 cells SLN-DNA vectors enter mainly by clathrin-mediated endocytosis (Figure 6H). However, the SAP vectors use caveolae/raft-dependent endocytosis to a greater extent (Figure 6E). Thus, in HEK293, SAP improves transfection via greater uptake of vectors. An SAP:DNA ratio of 50:1 was sufficient to provide this increase, as demonstrated in the higher percentage of EGFP positive cells; higher concentrations of SAP did not enhance transfection. In ARPE-19 cells, incorporation of SAP can favour caveolae/raft-dependent endocytosis over clathrin endocytosis.

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43 Nabi IR, Le PU. Caveolae/raft-dependent endocytosis. J Cell Biol. 2003; 161: 673-677.

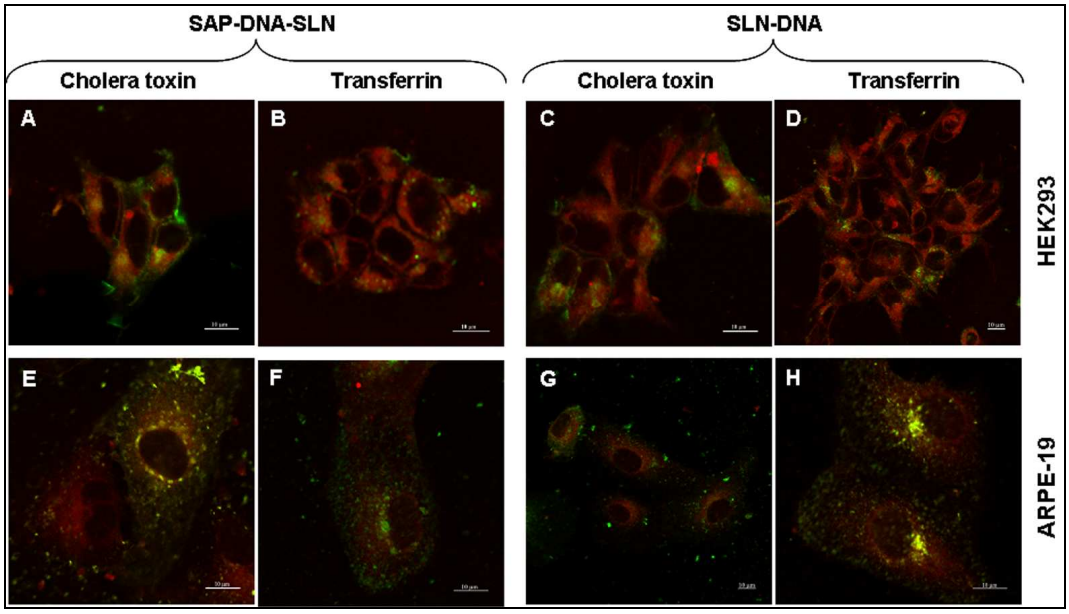

Figure 6. CLSM images of HEK293 cells (A to D) and ARPE-19 cells (E to H) co-incubated for 1 h with SLN-DNA vectors (C, D, G, H) or SAP-DNA-SLN vectors (A, B, E, F) and AlexaFluor488-cholera toxin at 10 μg/mL (A, C, E, G) or AlexaFluor488-Transferrin at 50μg/mL (B, D, F, H). The SLN:DNA ratio (w/w) was 5:1, and the SAP:DNA ratio (w/w) was 100:1. Nile Red-labelled vectors in red, AlexaFluor488-Cholera toxin or AlexaFluor488-Transferrin in green.

In order to confirm a change of the endocytosis pathway from clathrin to caveolae/raft endocytosis in ARPE-19 cells, we investigated the cell uptake of the vectors by co-incubating them with either filipin III, the caveolae/raft transport inhibitor, or chlorpromazine, the clathrin transport inhibitor. Filipin III binds to cholesterol, component of glycolipid microdomains and caveolae, and disrupt caveolar structure and function. Chlorpromazine inhibits receptor-mediated endocytosis by reducing the number of coated pit-associated receptors at the cell surface and causing the accumulation of clathrin and AP-2 in an endosomal compartment[39]. The inhibition experiments in ARPE-19 cells were previously optimized, since the inhibition is related to the type of cell, incubation time and

concentration of the applied inhibitor[44]. The concentration of inhibitors and incubation periods were selected based on cell viability and changes in cell volume and granularity, as measured by flow cytometry. Therefore, all experiments were accompanied by cell viability assays. Addition of either filipin III (1 μg/mL) or chlorpromazine (20 μg/mL) concomitantly with SLN-DNA vectors produced no inhibition and 10% inhibition in the particle uptake, respectively. When the uptake of SAP-DNA-SLN vectors was assayed in the presence of filipin III or chlorpromazine, 13% inhibition and no inhibition was achieved, respectively. Although modest inhibition was detected, when higher concentrations of inhibitors or longer periods of incubation were used reduction in cell viability and changes in cell volume and granularity were observed, as detected by flow cytometry (FSC/SCC parameters). It has to be taken in mind that due to the nature of biological systems, several dynamic processes might take place in parallel which might turn in compete with one another. Therefore, we focus the discussion of our results on qualitative interpretation more than quantitative data. Huth et al.[44] have also combined qualitative and quantitative analysis of liposomal-based systems entry, reaching similar conclusions about the focus on qualitative interpretation of the results.

Therefore, the use of the endocytosis inhibitors, as well as the co-localization study, suggests the change in the internalization mechanism from clathrin to caveolae/raft endocytosis in ARPE-19 cells when SAP was incorporated in the vectors. For targeted drug delivery, the mechanism of caveolae uptake seems to be most promising since it reduces lysosomal activity derived from clathrin-mediated endocytosis and localizes the vectors around the nucleus, facilitating their internalization when cells divide. This is

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44 Huth US, Schubert R, Peschka-Suss R. Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging. J Control Release. 2006; 110: 490-504.

consistent with the higher protein expression achieved with the formulations containing SAP.

Finally, although SAP led to higher transfection in ARPE-19 cells, even greater efficacy would be needed to treat retinal diseases. One strategy to improve transfection would be to facilitate nuclear internalization of DNA, which could be achieved via incorporation of nuclear localization signals into the peptide sequence[45].

4. CONCLUSIONS

SLN-DNA vectors containing SAP have greater transfection efficacy than SLN-DNA vectors alone in both HEK293 cells and ARPE-19 cells: in the former, by increasing the uptake of the vectors, and in the latter, by favouring caveolae/raft-dependent endocytosis over clathrin endocytosis. However, the extent to which one entry mechanism or the other is used is highly dependent on the amount of SAP in the formulation.

ACKNOWLEDGEMENTS

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SHORT- AND LONG-TERM STABILITY STUDY OF LYOPHILIZED SOLID LIPID NANOPARTICLES FOR GENE THERAPY

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ABSTRACT

Most studies in gene therapy are focused on developing more efficient non-viral vectors, ignoring their stability, even though physically and chemically stable vectors are necessary to achieve large easily shipped and stored batches. In the present work, the effect of lyophilization on the morphological characteristics and transfection capacity of solid lipid nanoparticles (LyoSLN) and SLN-DNA vectors (Lyo(SLN-DNA)) has been evaluated. The lyophilized preparations were stored under three different sets of temperature and humidity ICH conditions: 25ºC/60%RH, 30ºC/65%RH and 40ºC/75%RH. After lyophilization we found an increase in particle size which did not imply a reduction of "*in vitro*" transfection capacity. Stability studies of formulations lyophilized with trehalose showed that SLNs were physically stable during 9 months at 25ºC/60%RH and 6 months at 30ºC/65%RH. This stability was lost when harder conditions were employed (40ºC/75%RH). LyoSLN maintained or increased the transfection efficacy (from 19% to approximately 40% EGFP positive cells) over time only at 25ºC/60%RH and 30ºC/65%RH. Lyo(SLN-DNA) resulted in almost no transfection under all conditions. LyoSLN showed less DNA condensation capacity, whereas in Lyo(SLN-DNA) the plasmid became strongly bound, hampering the transfection. Furthermore, the storage of lyophilized lipoplexes stabilized with the disaccharide trehalose did not affect cell viability.

Keywords: gene therapy; non-viral vectors; solid lipid nanoparticles; lyophilization; stability study.

1. INTRODUCTION

The development of gene therapy has boosted the use of a new group of pharmaceutical agents for the treatment of human diseases[1,2,3]: gene delivery systems. Non-viral vectors are being extensively studied because of their greater security as compared to viral vectors. Non-viral vectors can be composed by polymers, lipids, peptides or mixtures of them. The typically employed lipidic systems are cationic liposomes, but it has been proved that cationic solid lipid nanoparticles (SLNs) can also provide good transfection levels[4,5,6]. Although the most well-known problem of non-viral systems is their low transfection efficiency, the poor physical stability of these systems in aqueous suspensions is also a barrier to their development as medicaments[7]. Lipoplexes tend to form aggregates which decrease their transfection capacity. In order to avoid this limitation, clinical trials using lipoplexes have traditionally used systems freshly prepared at the bedside prior to injection[8,9]. However, the quality and the particle size of these

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1 Jackson DA, Juranek S, Lipps HJ. Designing nonviral vectors for efficient gene transfer and long-term gene expression. Mol Ther. 2006; 14: 613-626.

2 Odom GL, Gregorevic P, Chamberlain JS. Viral-mediated gene therapy for the muscular dystrophies: Successes, limitations and recent advances. Biochim Biophys Acta. 2007; 1772: 243-262.

3 van der Wouden EA, Sandovici M, Henning RH, de Zeeuw D, Deelman LE. Approaches and methods in gene therapy for kidney disease. J Pharmacol Toxicol Meth. 2004; 50: 13-24.

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6 Olbrich C, Bakowsky U, Lehr CM, Müller RH, Kneuer C. Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA. J Control Release. 2001; 77: 345-355.

7 Anchordoquy TJ, Koe GS. Physical stability of nonviral plasmid-based therapeutics. J Pharm Sci. 2000; 89: 289-296.

8 Caplen NJ, Alton EWFW, Middleton PG, Dorin JR, Stevenson BJ, Gao X, et al. Liposome-mediated Cftr gene-transfer to the nasal epithelium of patients with cystic-fibrosis. Nat Med. 1995; 1: 39-46.

extemporaneous preparations are hardly ever controlled. Most studies in gene therapy are focused on developing more efficient non-viral vectors, ignoring their stability, even though physically and chemically stable vectors are necessary to achieve large easily shipped and stored batches.

Different authors[9,10,11,12] have demonstrated that frozen DNA formulations maintain transfection rates, but they require strict storage and shipping temperatures, which imply a substantial increase in costs. This fact has generated an increasing interest in developing dehydrated formulations, which can be stored and shipped at room temperatures. Lyophilization is one of the most employed techniques to generate dried pharmaceuticals in general, and DNA-based formulations in particular[9,12,13,14,15,16,17].

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9 Nabel GJ, Nabel EG, Yang ZY, Fox BA, Plautz GE, Gao X, et al. Direct gene-transfer with DNA liposome complexes in melanoma - Expression, biologic activity, and lack of toxicity in humans. P Natl Acad Sci USA. 1993; 190: 11307-11311.

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11 Cherng J-Y, Wetering P, Talsma H, Crommelin DJA, Hennink WE. Stabilization of polymer-based gene delivery systems Int J Pharm. 1999; 183: 25-28.

12 Hinrichs WLJ, Mancenido FA, Sanders NN, Braeckmans K, De Smedt SC, Demeester J, et al. The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying. Int J Pharm. 2006; 311: 237-244.

13 Allison SD, Molina M, Anchordoquy TJ. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. Biochim Biophys Acta. 2000; 1468; 127-138.

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15 Brus C, Kleemann E, Aigner A, Czubayko F, Kissel T. Stabilization of oligonucleotide-polyethylenimine complexes by freeze-drying: physicochemical and biological characterization. J Control Release. 2004; 95: 119-131.

16 Li B, Li S, Tan YD, Stolz DB, Watkins SC, Block LH, et al. Lyophilization of cationic lipid-protamine-DNA (LPD) complexes. J Pharm Sci. 2000; 89: 355-364.

Lyophilization subjects formulations to two important stresses, freezing and drying[7], which can damage biomolecules unless appropriate stabilizers, such as sugars, are used. Different sugars have been employed to stabilize lyophilized SLNs and non-viral vectors composed by cationic lipids and DNA[13,18,19,20,21]: monosaccharides (glucose), disaccharides (trehalose, sucrose), oligosaccharides (inuline) or polysaccharides (hydroxyethyl starch, high molecular weight dextrans). Among these, trehalose is one of the most commonly employed sugars providing positive results.

Although there are some studies about the long-term stability of SLNs for poorly water-soluble pharmaceutical drugs[22,23,24], we have not found any work about the long-term stability of lyophilized cationic SLNs for gene therapy, and only a few studies about

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17 Zhong ZR, Zhang ZR, Liu J, Deng Y, Zhang HW, Fu Y, et al. Characteristics comparison before and after lyophilization of transferrin modified procationic-liposome-protamine-DNA complexes (Tf-PLPD). Arch Pharm Res. 2007; 30: 102-108.

18 Allison SD, Anchordoquy TJ. Mechanisms of protection of cationic lipid-DNA complexes during lyophilization. J Pharm Sci. 2000; 89: 682-691.

19 Hinrichs WLJ, Sanders NN, De Smedt SC, Demeester J, Frijlink HW. Inulin is a promising cryo- and lyoprotectant for PEGylated lipoplexes. J Control Release. 2005; 103: 465-479.

20 Schwarz C, Mehnert W. Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles (SLN). Int J Pharm. 1997; 157: 171-179.

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22 Freitas C, Müller RH. Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN(TM)) dispersions. Int J Pharm. 1998; 168: 221-229.

23 Luo Y, Chen D, Ren L, Zhao X, Qin J. Solid lipid nanoparticles for enhancing vinpocetine's oral bioavailability. J Control Release. 2006; 114: 53-59.

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lyophilized liposomes[12,19,24,25,26]. The aim of our work was to evaluate the short- and long-term stability of lyophilized SLN containing the pCMS-EGFP plasmid. The lyophilized preparations were stored under three different sets of temperature and humidity conditions, according to ICH guidelines (CPMP/ICH/2736/99): 25ºC ± 2ºC/60%RH ± 5%RH (long-term study), 30ºC ± 2ºC/65%RH ± 5%RH (intermediate study) and 40ºC ± 2ºC/75%RH ± 5%RH (accelerated or short-term study). Parameters such as size, zeta potential, DNA protection and conformation, transfection capacity and cell viability were studied over time.

2. MATERIALS AND METHODS

2.1 Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTAP), lauryl sulfate sodium (SDS), glucose, antibiotic/antimycotic and D-trehalose were purchased from Sigma-Aldrich (Madrid, Spain). Tween-80 was provided by Vencaser (Bilbao, Spain) and dichloromethane by Panreac (Barcelona, Spain).

Plasmid pCMS-EGFP encoding the enhanced green fluorescent protein (EGFP) was purchased from BD Biosciences Clontech (Palo Alto, US) and amplified by Dro Biosystems S.L. (San Sebastián, Spain).

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25 Maitani Y, Aso Y, Yamada A, Yoshioka S. Effect of sugars on storage stability of lyophilized liposome/DNA complexes with high transfection efficiency. Int J Pharm. 2008; 356: 69-75.

26 Molina M, Anchordoquy TJ. Degradation of lyophilized lipid/DNA complexes during storage: The role of lipid and reactive oxygen species. Biochim Biophys Acta. 2008; 1778: 2119-2126.

The materials employed for the electrophoresis on agarose gel were acquired from Bio-Rad (Madrid, Spain). The cell culture reagents were purchased from LGC Promochem (Barcelona, Spain). The BD Viaprobe kit was provided by BD Biosciences (Belgium).

2.2 SLN and SLN-DNA vectors preparation

The SLNs were produced by a solvent emulsification/evaporation technique[27]. Precirol® ATO 5 was dissolved in dichloromethane (5% w/v), and then emulsified in an aqueous phase that contained DOTAP (0.4% w/v) and Tween-80 (0.1% w/v). The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) during 30 sec at 50 W. The organic phase:aqueous phase ratio was 1:5. Dichloromethane was then removed from the emulsion by evaporation using a magnetic agitator for 45 min followed by vacuum conditions for 15 min. A SLN suspension was formed upon precipitation of the Precirol® ATO 5 in the aqueous medium. Finally, the SLN were washed by centrifugation (3000 rpm, 20 min, x 3) using Millipore (Madrid, Spain) Amicon® Ultra centrifugal filters (100,000 MWCO).

SLN-DNA vectors were prepared by mixing during 30 min at 25ºC a pCMS-EGFP plasmid DNA solution at 2 μg/μL concentration and a SLN suspension, at DOTAP:DNA ratio 5:1 (w/w).

The formulations studied were the following: LyoSLNs, (LyoSLN)-DNA and Lyo(SLN-DNA). Table 1 summarizes how those formulations were prepared. In all cases, three batches were studied.

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27 Mehnert W, Mader K. Solid lipid nanoparticles - Production, characterization and applications. Adv Drug Deliv Rev. 2001; 47: 165-196.

Table 1. Names of the assayed formulations and summary of their preparation.

Name	Preparation
LyoSLNs	free DNA lyophilized SLNs and later reconstituted
(LyoSLN)-DNA	LyoSLNs later reconstituted and bound to DNA
Lyo(SLN-DNA)	lyophilized SLN-DNA vectors and later reconstituted

2.3 Lyophilization procedure

SLNs (without DNA) or SLN-DNA samples were transferred to poly-propilene tubes and diluted with the sugar solutions. Samples were frozen at -80ºC. After 24 h, frozen samples were lyophilized at -55ºC and 0.2 mbar for 48 h (Telstar Cryodos freeze-dryer). Lyophilized samples were reconstituted in HBS (Hepes Buffer Saline) by mixing, and the external appearance of the resulting formulations was observed.

2.4 Stability study conditions

LyoSLNs were packed in amber-colored borosilicate vials and the Lyo(SLN-DNA) in poly-propylene cryovials in order to prevent the DNA from binding to glass[28,29]. The samples were stored in environmental simulation chambers for constant climatic conditions (Binder, Tuttlingen, Germany). Table 2 shows the storage conditions used in the stability study, as well as the times when the samples were tested.

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28 Nanassy OZ, Haydock PV, Reed MW. Capture of genomic DNA on glass microscope slides. Anal Biochem. 2007; 365: 240-245.

29 Vogelstein B, Gillespie D. Preparative and analytical purification of DNA from agarose. P Natl Acad Sci USA. 1979; 76: 615-619.

Table 2. Storage conditions of the different groups included in the study (RH: relative humidity).

Group	Storage conditions	Time points
Time 0	---	0
Long-term	25ºC ± 2ºC/60%RH ± 5%RH	3, 6, 9 and 12 months
Intermediate	30ºC ± 2ºC/65%RH ± 5%RH	3, 6, 9 and 12 months
Accelerated	40ºC ± 2ºC/75%RH ± 5%RH	1, 3 and 6 months

2.5 Size and zeta potential measurements

The sizes of SLN and SLN-DNA vectors were determined by photon correlation spectroscopy (PCS). Zeta potential was measured by laser doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in 0.1 mM NaCl.

2.6 Agarose gel electrophoresis

SLN-DNA vectors were diluted in water Milli-Q™ to a final concentration of 0.1 μg DNA/μL and subjected to electrophoresis on an agarose gel (1% ethidium bromide included for visualization) for 30 min at 120 V. The bands were observed with a model TFX-20M transilluminator (Vilber-Lourmat). Images were captured using a digital camera from Bio-Rad, DigiDoc model.

2.7 SDS-induced release of DNA from SLN-DNA vectors

A 2% SDS solution was added to the samples to a final concentration of 1% to release DNA from SLN. Samples were then analysed by electrophoresis on agarose gel and the integrity of the DNA in each sample was compared with untreated DNA as control.

2.8 DNase I protection study

DNase I was added to SLN-DNA systems to a final concentration of 1 U DNase I/2.5 μg DNA, and the mixtures were incubated at 37ºC for 30 min. Afterwards, 2% SDS solution was added to the samples to a final concentration of 1% to release DNA from SLN. Samples were then analysed by electrophoresis on agarose gel and the integrity of the DNA in each sample was compared with untreated DNA as control.

2.9 Cell culture and transfection protocol

"*In vitro*" assays were performed with the Human Embryonic Kidney (HEK293) cell line, obtained from the American Type Culture Collection (ATCC). Cells were maintained in Eagle´s Minimal Essential Medium with Earle´s BSS and 2 mM L-glutamine (EMEM) supplemented with 10% heat-inactivated horse serum and 1% antibiotic/antimycotic. Cells were incubated at 37ºC with 5% CO_2 in air and subcultured every 2-3 days using trypsin/EDTA.

For transfection, HEK293 cells were seeded on 24 well plates at a density of 150,000 cells per well and allowed to adhere overnight. 75 μL of the vectors solution diluted in HBS buffer (2.5 μg DNA) was added, and cells were incubated with the non-viral systems for 4 h at 37ºC. The medium containing the vectors in the wells was diluted with 1 mL of complete medium, and cells were allowed to grow for 72 h.

2.10 Flow cytometry-mediated analysis of transfection efficacy and cytotoxicity

At the end of the incubation period, cells were washed once with 300 μL of PBS and detached with 300 μL of trypsin/EDTA. Then the cells were centrifuged at 1500g and the supernatant was discarded. The cells were resuspended with PBS and directly introduced to a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, USA). For each sample, 10,000 events were collected.

For transfection, efficacy quantitation fluorescence of EGFP was collected at 525 nm (FL1). For cytotoxicity measurements, BD Via-Probe kit was employed. 5 μL of the kit was added to each sample and after 10 min of incubation fluorescence correspondent to dead cells was measured at 650 nm (FL3).

2.11 Statistical analysis

Results are reported as means (S.D. = standard deviation). The statistical analysis was carried out with SPSS 14.0 for Windows® (SPSS®, Chicago, USA). Normal distribution of samples was assessed by the Shapiro–Wilk's test, and homogeneity of the variance by the Levene's test. The statistical analysis between non-lyophilized and lyophilized samples or between lyophilized samples at different times was determined with a Student´s t-test. Differences were considered statistically significant if $p < 0.05$.

3. RESULTS

3.1 Characterization of lyophilized samples at time 0

3.1.1 Physical appearance

Lyophilization of samples without stabilizers resulted in the aggregation of particles forming a cake with rubbery aspect. When LyoSLNs were lyophilized with a solution of glucose at 5%, a powered product was obtained, but after 2 days at room temperature it became in rubber. The use of the other glucose concentrations led to rubbery samples from the beginning. But when trehalose was used as stabilizer at 10 and 5% concentrations, lyophilized samples showed a powdery aspect. However, when trehalose solutions at 40 and 20% were used, samples showed a fibrous aspect. Redispersion in HBS buffer by vortex agitation was rapid in all cases, although those stabilized with 5% trehalose presented microparticles.

3.1.2 Size and zeta potential

Figure 1 shows the effect of trehalose concentration on the particle size and zeta potential of free DNA LyoSLN (Figure 1A) and Lyo(SLN-DNA) (Figure 1B) formulations.

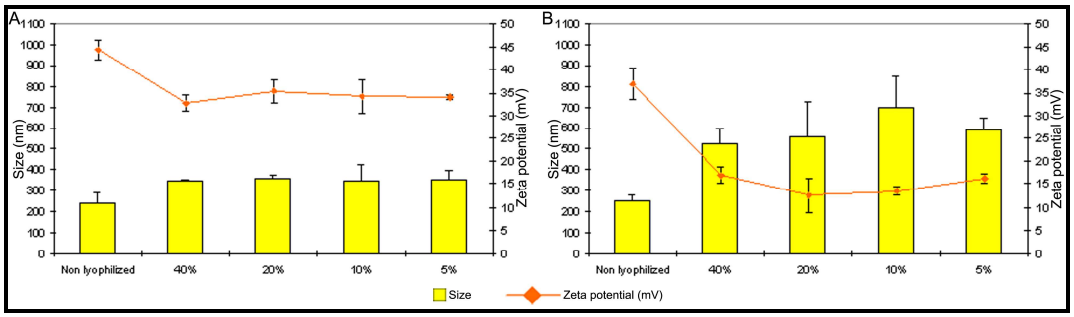

Figure 1. Effect of trehalose concentration on size and zeta potential of LyoSLNs (A) and Lyo(SLN-DNA) (B). X axe indicates the concentration of trehalose solutions employed (w/v). Error bars represent S.D. (n=3). *: $p < 0.01$ in zeta potential against non-lyophilized samples; **: $p < 0.05$ in zeta potential against non-lyophilized samples; ##: $p < 0.05$ in size against non-lyophilized samples.

Lyophilization of SLNs (LyoSLNs) caused an increase in the particle size (bars in Figure 1A) of approximately 100 nm with all trehalose concentrations ($p < 0.05$). Zeta potential (line in Figure 1A) decreased from +44 mV to approximately +35 mV in all cases ($p < 0,05$). When (LyoSLN)-DNA vectors were prepared, the size was higher than 1 μm, and the zeta potential significantly decreased to values lower than +10mV.

Concerning Lyo(SLN-DNA), lyophilization induced a 2-fold increase in size (bars in Figure 1B). Zeta potential (line in Figure 1B) decreased to around +15 mV with all the trehalose concentrations ($p < 0.05$).

3.1.3 Binding of DNA to SLN

The agarose gel electrophoresis in Figure 2 (time 0) shows that LyoSLNs lyophilized in the presence of trehalose solutions at 10 and 5% (lanes 2 and 3, respectively) had the ability to bind all DNA, as no band was detected in those lanes.

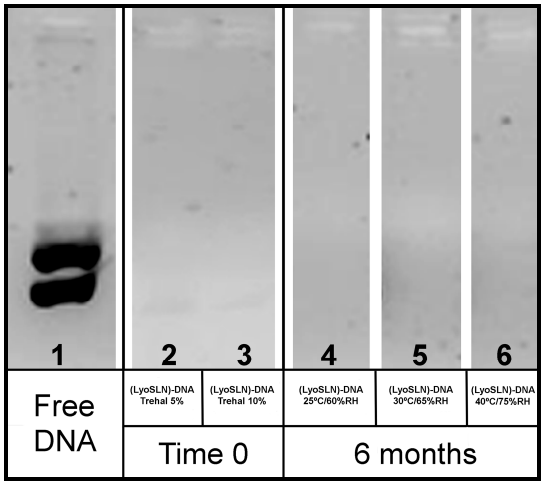

Figure 2. Agarose gel electrophoresis of DNA in contact with LyoSLNs at time 0 and after 6 months of storage. DOTAP:DNA ratio was 5:1 (w/w). Lane 1: free DNA, lane 2: DNA bound to LyoSLNs lyophilized in presence of a trehalose 5% solution at time 0, lane 3: DNA bound to LyoSLNs lyophilized in presence of a trehalose 10% solution at time 0, lane 4: DNA bound to LyoSLNs lyophilized in presence of a trehalose 10% solution at 6 months of storage at 25ºC/60%RH, lane 5: DNA bound to LyoSLNs lyophilized in presence of a trehalose 10% solution at 6 months of storage at 30ºC/65%RH, lane 6: DNA bound to LyoSLNs lyophilized in presence of a trehalose 10% solution at 6 months of storage at 40ºC/75%RH.

3.1.4 SDS-induced release of DNA from lyophilized samples

Figure 3 shows the agarose gel electrophoresis corresponding to the DNA released from lyophilized formulations in the presence of trehalose 5 and 10% at time 0 (lanes 2-5). The DNA released from (LyoSLN)-DNA (lanes 2 and 3) showed two bands, which correspond to the open circular conformation (OC) and the super-coiled conformation (SC). Those bands fit with the bands of the free DNA (lane 1). However, the DNA released from Lyo(SLN-DNA) showed three bands (lanes 4 and 5), with the intermediate band as the linear DNA (L).

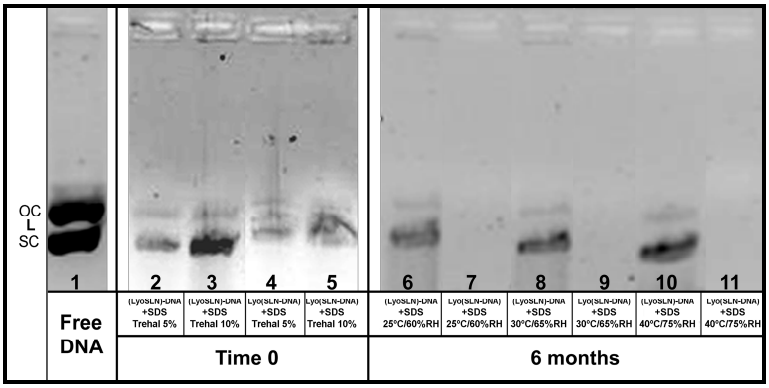

Figure 3. Agarose gel electrophoresis of (LyoSLN)-DNA and Lyo(SLN-DNA) treated with SDS 1%. Lane 1: free DNA, lane 2: DNA released from LyoSLNs lyophilized in presence of a trehalose 5% solution at time 0, lane 3: DNA released from LyoSLNs lyophilized in presence of a trehalose 10% solution at time 0, lane 4: DNA released from Lyo(SLN-DNA) lyophilized in presence of a trehalose 5% solution at time 0, lane 5: DNA released from Lyo(SLN-DNA) lyophilized in presence of a trehalose 10% solution at time 0, lane 6: DNA released from LyoSLNs lyophilized in presence of a trehalose 10% solution at 6 months of storage at 25ºC/60%RH, lane 7: DNA released from Lyo(SLN-DNA) lyophilized in presence of a trehalose 10% solution at 6 months of storage at 25ºC/60%RH, lane 8: DNA released from LyoSLNs lyophilized in presence of a trehalose 10% solution at 6 months of storage at 30ºC/65%RH, lane 9: DNA released from Lyo(SLN-DNA) lyophilized in presence of a trehalose 10% solution at 6 months of storage at 30ºC/65%RH, lane 10: DNA released from LyoSLNs lyophilized in presence of a trehalose 10% solution at 6 months of storage at 40ºC/75%RH, lane 11: DNA released from Lyo(SLN-DNA) lyophilized in presence of a trehalose 10% solution at 6 months of storage at 40ºC/75%RH. OC: open circular DNA, L: lineal DNA, SC: super-coiled DNA.

3.1.5. Transfection "*in vitro*" and cell viability

Figure 4 shows transfection and cell viability of non-lyophilized and lyophilized formulations in HEK293 culture cells. Assays were carried out with lyophilized samples stabilized with 5 or 10% trehalose solutions. No differences in transfection levels (bars) were detected between non-lyophilized and lyophilized samples ($p > 0.05$). Furthermore, cell viability (line) was not modified by the lyophilization process ($p > 0.05$).

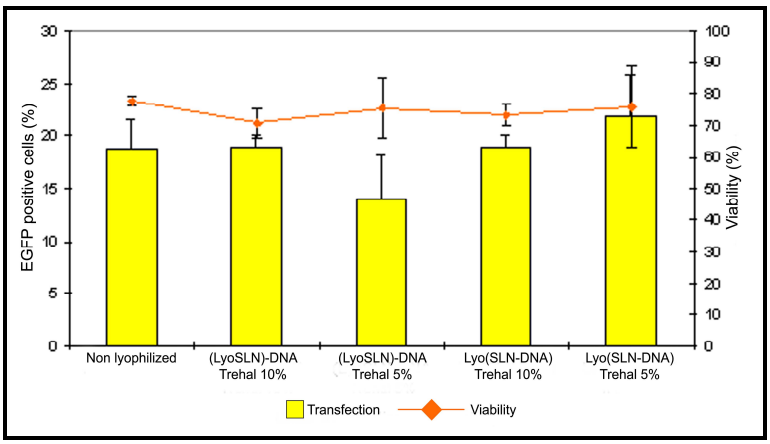

Figure 4. Transfection and cell viability of non-lyophilized and lyophilized formulations in presence of trehalose solutions at 5 or 10% concentrations. Error bars represent S.D. (n=3). DOTAP:DNA 5:1 (w/w).

3.2. Stability of lyophilized samples over time

For the stability assay, 10% trehalose solution was chosen as stabilizer. Samples were stored at different temperature and humidity conditions and assayed at different times, as it is summarized in Table 2.

3.2.1. Physical appearance over time

LyoSLNs showed a powdered aspect and were easily dispersed in HBS buffer until 6 months at 30ºC/65%RH and until 9 months at 25ºC/60%RH. The samples stored at 40ºC/75%RH maintained that powered aspect but after redispersion they showed

aggregates. Lyo(SLN-DNA) showed a similar behaviour maintaining the powered aspect and the dispersion capacity during 6 and 12 months at 30ºC/65%RH and 25ºC/60%RH, respectively. After storage at 40ºC/75%RH, Lyo(SLN-DNA) presented a rubbery aspect and inadequate redispersion from the first month.

3.2.2. Size and zeta potential over time

Particle size and zeta potential of the LyoSLNs and Lyo(SLN-DNA) were measured at different time points. Figure 5 features the results.

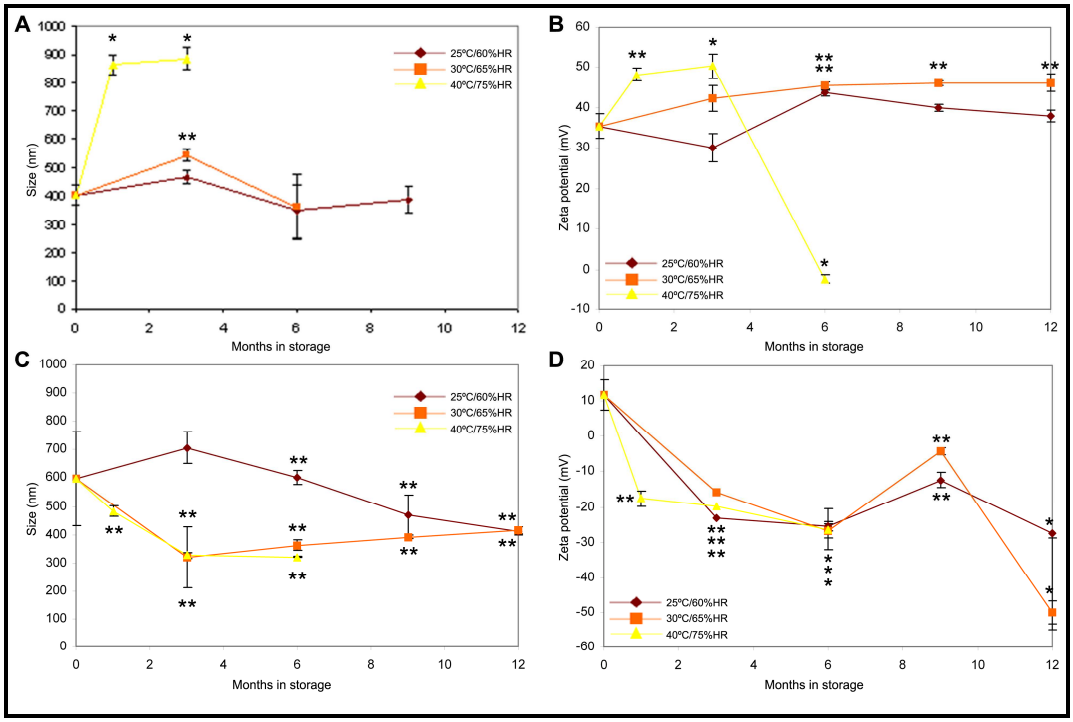

Figure 5. Size and zeta potential of LyoSLNs (A, B) and Lyo(SLN-DNA) (C, D) stored at different conditions during different times from 1 to 12 months (n=3). X axe indicates time in storage (months). Error bars represent S.D. (n=3). *: $p < 0.01$ against time 0; **: $p < 0.05$ against time 0.

LyoSLNs stored at 40ºC/75%RH for 1 and 3 months showed sizes (Figure 5A) twice greater than that at time 0 ($p < 0.01$), and at 6 months we visually detected aggregates,

unmeasured by PCS. At 30ºC/65%RH and 25ºC/60%RH, particle size hardly varied during the first 6 and 9 months, respectively. From these times, redispersion was not complete and aggregates appeared. Zeta potential (Figure 5B) was stable when formulations were stored at 25ºC/60%RH, although a slight but significant increase in surface charge (about 10 mV) was found when storing at 30ºC/65%RH. At 40ºC/75%RH zeta potential increased during 1 and 3 months, but when measured after 6 months it decreased to -2.3 mV.

As to Lyo(SLN-DNA) formulations (Figure 5C), during the 12-month storage period, a decrease in size from 700 nm to 450 nm was observed at all storage conditions (p<0,05). Nevertheless, this reduction was progressive at 25ºC/60%RH, but at 30ºC/65%RH and 40ºC/75%RH maximum reduction was obtained at 3 months. Zeta potential (Figure 5D) decreased from +12 mV to negative values in all cases (p<0.05).

3.2.3 Binding of DNA to stored LyoSLN

Figure 2 (lanes 4-6) features the results of the gel electrophoresis carried out after a 6-month storage period. The absence of bands indicates that LyoSLNs maintained the DNA binding capacity during the storage at the three temperature and humidity conditions. That capacity was maintained throughout the study.

3.2.4. SDS-induced release of DNA from stored lyophilized samples

Figure 3 shows the agarose gel electrophoresis of (LyoSLN)-DNA and Lyo(SLN-DNA) treated with SDS 1% after 6 months at the three conditions of temperature and humidity. Only lanes 6, 8 and 10, corresponding to (LyoSLN)-DNA, showed free DNA. Lyo(SLN-DNA) formulations did not release the DNA (lanes 7, 9 and 11). This behaviour was observed during all the study.

3.2.5. "*In vitro*" protection against DNase I

Figure 6 shows the agarose gel electrophoresis of the (LyoSLN)-DNA lipoplexes treated with DNase I. Lane 1 represents not-treated free DNA. Lanes 2, 3 and 4 correspond to DNA released from (LyoSLN)-DNA stored at the three conditions of temperature and humidity at 6 months, treated with DNase I, and lane 5 shows the DNA released from LyoSLNs when stored at 40ºC/75%RH for 3 months, after the treatment with DNase I. Lanes 2, 3 and 5 show a less intense SC band, the appearance of the L band and DNA fragmentation. These results indicate that LyoSLNs were still able to partially protect the DNA; this behaviour was observed throughout the study at 25ºC/60%RH and 30ºC/65%RH. Nevertheless, the DNA protection was totally lost after a 6-month storage period at 40ºC/75%RH, as can be seen in lane 4, where all DNA is fragmented.

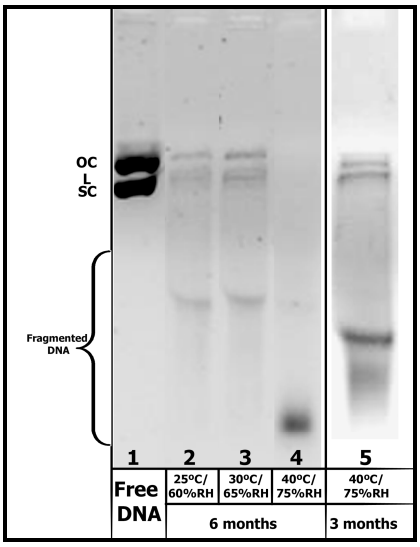

Figure 6. Agarose gel electrophoresis of the (LyoSLN)-DNA complexes treated with DNase I. Lane 1: free DNA, lane 2: (LyoSLN)-DNA lyophilized in presence of a trehalose 10% solution at 6 months of storage at 25ºC/60%RH, lane 3: (LyoSLN)-DNA lyophilized in presence of a trehalose 10% solution at 6 months of storage at 30ºC/65%RH, lane 4: (LyoSLN)-DNA lyophilized in presence of a trehalose 10% solution at 6 months of storage at 40ºC/75%RH, lane 5: (LyoSLN)-DNA lyophilized in presence of a trehalose 10% solution at 3 months of storage at 40ºC/75%RH. OC: open circular DNA, L: lineal DNA, SC: super-coiled DNA.

3.2.6. "*In vitro*" transfection and cell viability over time

Figure 7 presents the percentage of transfected cells and cell viability measured by flow cytometry.

After 6 months, the percentage of transfected cells with (LyoSLN)-DNA increased from 19% to 40% when stored at 25ºC/60%RH and to 37% when stored at 30ºC/65%RH, decreasing to initial values after 12 months. However, transfection levels suffered an important decrease to 1.5% EGFP positive cells at 6 months ($p < 0.01$) when (LyoSLN)-DNA formulations were stored at 40ºC/75%RH.

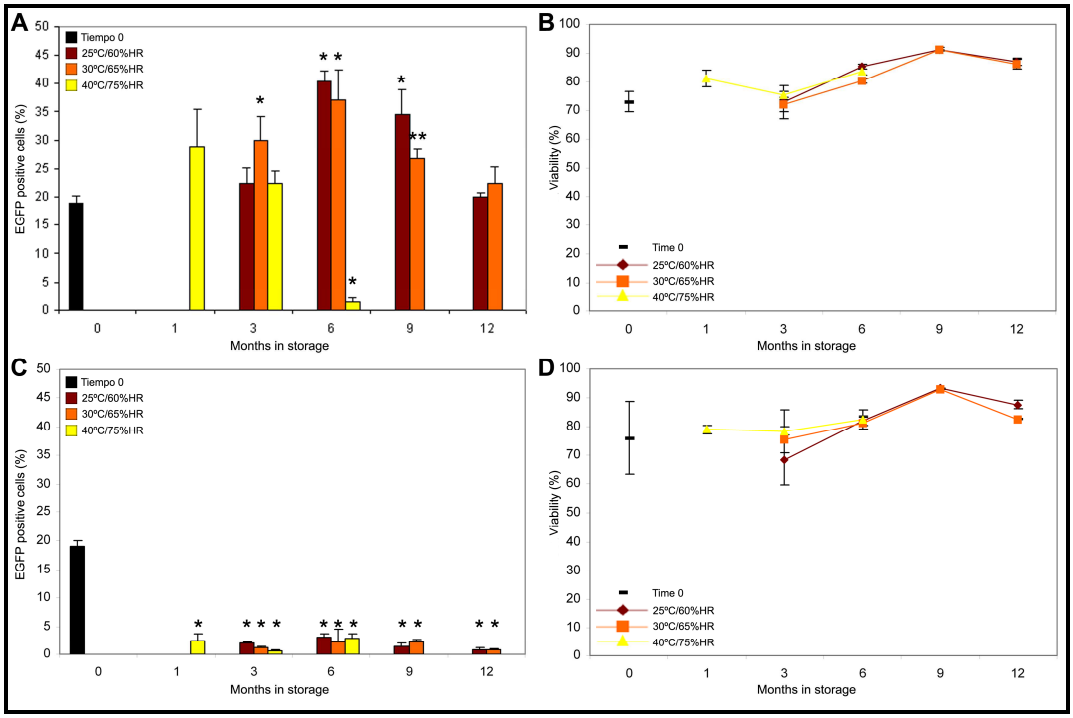

Figure 7. Transfection (graphics in the left) and cell viability (graphics in the right) of (LyoSLN)-DNA (A, B) and Lyo(SLN-DNA) (C, D) stored at different conditions along time (n=3). X axe indicates the months of storage. Error bars represent S.D. (n=3). *: $p < 0.01$ against time 0. **: $p < 0.05$ against time 0.

Lyo(SLN-DNA) resulted in a dramatically reduction of transfection efficiency in all storage conditions ($p < 0.01$), reaching percentages of EGFP positive cells under 3% (Figure 7C).

The lyophilization and posterior storage did not have an effect on cell viability in any case, (Figure 7B and 7D).

4. DISCUSSION

An important limitation of aqueous suspensions of non-viral vectors is their poor stability[7,30,31,32]. SLN suspensions are not an exception. In a previous work, Freitas and Müller[22] showed an increase in particle size of SLNs in a short period of time; although formulations prepared by these authors were not used for gene therapy, the same behaviour should be expected for our SLNs. In fact, the SLN suspensions employed in the present study, composed by Precirol® ATO 5, DOTAP and Tween-80, were stable only for 1 week at room temperature and for 1 month at 4ºC (data not shown).

Lyophilization is one of the most employed methods to obtain dehydrated formulations which can be stored and shipped at room temperatures. However, it subjects samples to two main transformations which results in additional stability problems. First, the aqueous dispersion is freezing, prior to the evaporation of water under vacuum. Then, and before the administration, re-dispersion must be done. The process of lyophilization of SLNs, with or without DNA and in absence of any stabilizer samples, resulted in the aggregation

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30 Gustafsson J, Arvidson G, Karlsson G, Almgren M. Complexes between cationic liposomes and DNA visualized by Cryo-Tem. Biochim Biophys Acta. 1995; 1235: 305-312.

31 Mahato RI. Non-viral peptide-based approaches to gene delivery. J Drug Targ. 1999; 7: 249-268.

32 Tang MX, Szoka FC. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Ther. 1997; 4: 823-832.

of particles forming a rubbery-looked cake. The freezing and the redispersion of the samples may be responsible for the aggregation because in both cases the water amount where particles are suspended decreases, which favours their concentration[13,27,33]. In the present work, the effect of lyophilization of SLNs and SLN-DNA vectors on their morphological characteristics and transfection capacity has been evaluated. The first strategy was to lyophilize the SLNs and later reconstitute them to finally prepare the lipoplexes with the plasmid. The second strategy was to lyophilize the lipoplexes, which would be much more convenient because it only requires the reconstitution prior to clinical use. As lyophilization can damage DNA[34], resulting in a loss of activity, an exhaustive evaluation of the behaviour of the lyophilized products is necessary. In order to improve stability, carbohydrates as lyoprotectants are frequently used, avoiding particle aggregation and denaturation of macromolecules.

Different studies have demonstrated that disaccharides are good stabilizers for lipoplexes[13,14,18], and one of them, trehalose, has been extensively demonstrated to provide good stabilization of lyophilized SLN[20,21,35,36]. The stabilizer sugar must possess high glass transition temperature (T_g) to maintain the temperature during lyophilization below it. Otherwise, the freeze concentrated fraction would be in the liquid or in the

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33 Molina MDC, Allison SD, Anchordoquy TJ. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: Insight from other structural indicators. J Pharm Sci. 2001; 90: 1445-1455.

34 Poxon SW, Hughes JA. The effect of lyophilization on plasmid DNA activity. Pharm Dev Technol. 2000; 5: 115-122.

35 Cavalli R, Caputo O, Carlotti ME, Trotta M, Scarnecchia C, Gasco MR. Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. Int J Pharm. 1997; 148: 47-54.

36 Heiati H, Tawashi R, Phillips NC. Drug retention and stability of solid lipid nanoparticles containing azidothymidine palmitate after autoclaving, storage and lyophilization. J Microencapsul. 1998; 15: 173-184.

rubbery state during the process[37]. Furthermore, the samples lyophilized in the presence of sugars with high T_g (trehalose) show less tendency to aggregate during storage as compared to sugars with lower T_g, such as sucrose or glucose[38]. When we used the monosaccharide glucose as stabilizer, the lyophilized samples showed a rubbery aspect. Only when LyoSLNs were lyophilized with a solution of glucose at 5% a powered product was obtained, but after 2 days at room temperature it became rubber. However, the lyophilization with the stabilizer trehalose provided fibrous or powdery samples. On the basis of these observations, we have chosen trehalose as stabilizer of SLN-based non-viral vectors.

Our results indicate that the stabilizer effect of trehalose on the physical appearance of LyoSLNs and Lyo(SLN-DNA) was similar. In both cases, the lyophilized samples showed a powdery aspect when 10 or 5% trehalose solutions were used. Moreover, redispersion by vortex agitation was rapid for all the trehalose concentrations studied, except for formulations stabilized with 5% trehalose, in which microparticles appeared.

When comparing the particle size and the zeta potential of the SLNs (Figure 1A), lyophilization induced a size increase (about 100 nm) and a slight decrease of zeta potential, regardless of the trehalose concentration. When those LyoSLNs were bound to DNA, vectors showed sizes bigger than 1 μm and superficial charge lower than +10 mV. When lipoplexes were lyophilized, Lyo(SLN-DNA), lyophilization caused a 2-fold or even higher increase in particle size. Moreover, a significant decrease in the zeta potential was

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37 Hinrichs WLJ, Prinsen MG, Frijlink HW. Inulin glasses for the stabilization of therapeutic proteins. Int J Pharm. 2001; 215: 163-174.

38 Molina MD, Armstrong TK, Zhang Y, Patel MM, Lentz YK, Anchordoquy TJ. The stability of lyophilized lipid/DNA complexes during prolonged storage. J Pharm Sci. 2004; 93: 2259-2273.

observed. Therefore, in both cases lyophilization induces changes on particles leading to a decrease in the zeta potential and to an increase in the size. The decrease in superficial charge could be attributed to two phenomena: the Schiff's base[19] or the water replacement hypothesis[39,40]. The Schiff's base phenomenon refers to the reaction between reducing groups of sugars and amine groups of DOTAP. This is possible when reducing sugars are employed, or when non-reducing sugars are hydrolyzed resulting in the formation of reducing ones. Nevertheless, trehalose is a non-reducing disaccharide which is strongly resistant to hydrolysis, so the Schiff's base would not be responsible for the reduction on the superficial charge. Therefore, the decrease in the zeta potential may be better explained by the formation of hydrogen bonds between trehalose and SLN-DNA vectors (water replacement hypothesis). Those bonds can happen with cationic lipid head groups or with DNA. The cationic lipid head groups have the ammonium groups, which are responsible for the positive charge. When sugars form hydrogen bonds with those groups, the positive charge is partially neutralized, the superficial charge decreases and the binding of DNA by electrostatic interactions is weaker, keeping less condensed. The smaller condensation results in an increase in the size and a decrease in the zeta potential, as the negative charges of DNA are more exposed.

As the decrease in the zeta potential may reduce the DNA binding capacity of the LyoSLNs, we carried out an agarose gel electrophoresis with (LyoSLN)-DNA lipoplexes (Figure 2), and we demonstrated that those formulations maintained the binding capacity,

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39 Crowe JH, Crowe LM. Preserving dry biomaterials: the water replacement hypothesis, Part 1. BioPharm. 1993; 6: 28-37.

40 Crowe JH, Crowe LM. Preserving dry biomaterials: the water replacement hypothesis, Part 2. BioPharm. 1993; 6: 40-43.

necessary for transfection. DNA topology is another factor which needs to be evaluated during the development of non-viral vectors. The agarose gel electrophoresis in Figure 3 shows the bands corresponding to the DNA released from the lyophilized formulations. When DNA was released from LyoSLNs (lanes 2 and 3 in Figure 3), the original bands of free DNA appeared. However, the DNA released from Lyo(SLN-DNA) showed three bands (lanes 4 and 5 in figure 3); an intermediate band corresponding to the linear DNA (L) appeared. The OC isoform is formed by a reversible change from SC DNA, and the L isoform appears when the OC isoform is degraded; that conversion is not reversible. Accordingly, lyophilization partially damaged DNA, although the most bioactive isoform[41,42], the SC DNA, did not completely disappear.

We also studied the transfection capacity of both (LyoSLN)-DNA and Lyo(SLN-DNA) formulations. Figure 4 shows that all formulations maintained transfection rates and cell viability. Several studies have correlated the maintenance of lipoplex size and transfection rate. In our study, lyophilization did not produce changes in cell transfection in spite of the increase in size; however, it is important to consider that in all these studies[14,16,18,33,38,43] liposomes but not SLNs were evaluated. Therefore, changes in the particle size do not necessary imply changes in the "*in vitro*" transfection efficacy. It is important to take into account that the change in particle size after lyophilization is an important factor to be considered when administering the vectors "*in vivo*" because it may

41 Middaugh CR, Evans RK, Montgomery DL, Casimiro DR. Analysis of plasmid DNA from a pharmaceutical perspective. J Pharm Sci. 1998; 87: 130-146.

42 Remaut K, Sanders NN, Fayazpour F, Demeester J, De Smedt SC. Influence of plasmid DNA topology on the transfection properties of dotap/dope lipoplexes. J Control Release. 2006; 115: 335-343.

43 Seville PC, Kellaway IW, Birchall JC. Preparation of dry powder dispersions for non-viral gene detivery by freeze-drying and spray-drying. J Gene Med. 2002; 4: 428-437.

compromise the administration route, the biodistribution and the efficacy. Size influences the targeting of the intravenously administered gene delivery systems, because the structure of the blood capillary wall varies greatly in different organs and tissues[44]. Furthermore, after oral administration the size of the particles determines the uptake by Peyer's patches and passage to blood[45]. Superficial charge of the gene delivery systems is also important, because it does not only determine the physical aspect, but it may also influence the "*in vivo*" behaviour. We have also observed in this work that despite the differences in the net charge of the SLN-DNA vectors before and after lyophilization, "*in vitro*" transfection levels did not undergo changes. However, an excess of positive charges when vectors are administered to living animals induces the binding to serum or plasma components such as proteins or erythrocytes, resulting in the elimination from bloodstream and a reduction in the efficacy[46].

Once it was demonstrated that the lyophilized formulations maintained the transfection capacity, a stability study was performed. Trehalose solution at 10% was chosen as lyoprotectant, because powdery samples were obtained, without the presence of microparticles after redispersion. The conditions and time of storage are summarized in Table 2.

During storage, stability of LyoSLNs depended on the conditions of temperature and humidity. The formulations maintained the particle size (Figure 5A) during 6 and 9

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44 Kawakami S, Higuchi Y, Hashida M. Nonviral approaches for targeted delivery of plasmid DNA and oligonucleotide. J Pharm Sci. 2008; 97: 726-745.

45 Delie F. Evaluation of nano- and microparticle uptake by the gastrointestinal tract. Adv Drug Deliv Rev. 1998; 34: 221-233.

46 Schiffelers RM, de Wolf HK, van Rooy I, Storm G. Synthetic delivery systems for intravenous administration of nucleic acids. Nanomedicine. 2007; 2: 169-181.

months, at 30ºC/65%RH and 25ºC/60%RH, respectively. From those times, LyoSLN suspensions became heterogeneous, with particles of 400 nm but also smaller nanoparticles of about 40 nm, indicating changes in SLNs. Zeta potential (Figure 5B) did not change at 25ºC/60%RH, but at 30ºC/65%RH it suffered a slight and progressive increase over time. The physical appearance of the samples stored at 40ºC/75%RH was rubbery from the first month and dispersion was difficult. The presence of particles with size twice higher than at time 0 resulted in unmeasured aggregates at 6 months, fitting with a sudden decrease in the zeta potential to negative values (-2 mV). As we have mentioned above, the samples lyophilized in the presence of sugars with high T_g, such as trehalose, show less tendency to aggregate during storage at temperatures quite below the T_g, as vectors possess more restricted mobility[47]. Therefore, in our opinion the physical changes in the vectors rather than aggregation should be responsible for increase in the size. Figure 7A shows that after 6 months, the percentage of transfected cells with (LyoSLN)-DNA increased from 19 to 40% when stored at 25ºC/60%RH and to 37% when stored at 30ºC/65%RH, decreasing to initial values after 12 months. The size does not seem to be the responsible for these differences, because when the formulations were stored at 25ºC/60%RH the increase in the transfection levels was observed at 6 and 9 months, and the differences in size were detected after 9 months of storage. Moreover, when samples were stored at 30ºC/65%RH the improvement in transfection was observed from 3 to 9 months, and the changes in size distribution occurred after 6 months of storage.

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47 Hancock BC, Shamblin SL, Zografi G. Molecular mobility of amorphous pharmaceutical solids below their glass-transition temperatures. Pharm Res. 1995; 12: 799-806.

In spite of the changes observed during the study, the DNA binding capacity was not lost (lanes 4-6 in Figure 2), although protection was reduced. The gel electrophoresis of the samples treated with DNase I (Figure 6) shows that the protection of DNA by LyoSLNs stored at 25ºC/60%RH and 30ºC/65%RH decreased, as the SC band was less intense and the L band and fragmented DNA appeared. We had previously observed[4] that the decrease in the protection is probably due to a lower DNA condensation, which favours the release of the plasmid improving transfection "*in vitro*". This lower DNA condensation, better than size, may be responsible for the higher transfection levels mentioned above. This could be an important drawback when using the formulations "*in vivo*", since a higher DNA release could facilitate the plasmid degradation and thus a reduction in transfection efficacy. Furthermore, variation in transfection efficacy over time (Figure 7A) could imply an erratic pharmacological response.

Formulations stored at 40ºC/75%RH lost their transfection capacity at 6 months probably due to the low DNA condensation, which is not only necessary to protect DNA but also to facilitate the mobility of DNA molecules through the cytoplasm to the nucleus. These formulations showed a too weak DNA condensation capacity (lane 4 in figure 6), necessary to protect and transport it through the cytoplasm. If condensation is not enough to direct DNA to the nucleus, posterior entry is hampered and consequently transfection decreases.

The storage of Lyo(SLN-DNA) in all conditions studied led to an important loss of transfection. In the SDS-induced DNA release study, no bands were observed in the gel electrophoresis (lanes 7, 9 and 11 in Figure 3). The decrease in the size and the absence of free DNA after treatment with SDS indicate that the bounds between SLN and DNA become stronger during storage. The release of DNA from vectors is necessary in the

cytoplasmic compartment for the posterior entry to the nucleus and synthesis of the encoded protein[4]. Therefore, the strong DNA binding to the SLNs seems to be responsible for the lack of transfection. Moreover, the negative values of zeta potential indicate that the charges of DNA are more exposed, in spite of the high condensation degree, indicating a different DNA disposition.

This work also shows that the storage of lyophilized lipoplexes stabilized with the disaccharide trehalose did not affect cell viability (Figure 7B and Figure 7D).

5. CONCLUSIONS

On the basis of these results, we can conclude that lyophilization with trehalose may provide physically stable dried SLNs during 6 months at 30ºC/65%RH and 9 months at 25ºC/60%RH. This stability was lost when harder conditions are employed (40ºC/75%RH). LyoSLNs maintained or increased the transfection efficacy over time at 25ºC/60%RH and 30ºC/65%RH, but not if they were stored at 40ºC/75%RH, and Lyo(SLN-DNA) resulted in almost no transfection in all conditions. Differences in DNA condensation between the two kinds of formulations explain the differences observed in the transfection efficacy. LyoSLNs showed less DNA condensation capacity, whereas in Lyo(SLN-DNA) the plasmid became strongly bound, hampering transfection. Unlike liposomes, the increase in size occurring during the lyophilization of SLNs does not imply a reduction in "*in vitro*" transfection capacity. This study shows that it is possible to prepare lyophilized SLNs easily stored in controlled conditions, although their "*in vivo*" application still requires some improvements in order to extend the stability period and to control variability in transfection efficacy.

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"*IN VIVO*" TRANSFECTION CAPACITY OF SOLID LIPID NANOPARTICLES (SLNs) AFTER INTRAVENOUS ADMINISTRATION TO MICE

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ABSTRACT

Naked plasmid DNA is a powerful tool for gene therapy, but it is rapidly eliminated from the circulation after intravenous administration. Therefore, it is necessary the development of optimized DNA delivery systems for its successful clinical use. Gene delivery using cationic lipids has gained increasing interest as an alternative for viral vectors. In this work we have studied the capacity of solid lipid nanoparticles (SLNs) to transfect "*in vivo*" after intravenous administration in mice. The SLNs, composed by Precirol® ATO 5, DOTAP and Tween-80 were complexed with the plasmid pCMS-EGFP which encodes the Enhanced Green Fluorescent Protein (EGFP). The resulting systems showed a mean particle size of 276 nm, superficial charge of +28 mV, were able to protect the plasmid and to transfect "*in vitro*", achieving 40% of EGFP positive HEK293 cells. The intravenous administration in mice led to transfection in hepatic tissue and spleen during 7 days, but not in lungs where non-viral vectors are commonly accumulated. We attribute the absence of transfection in lungs to the presence of PEG chains of Tween-80 which partially neutralize the positive charges of SLNs decreasing the formation of aggregates with blood components and the consequent accumulation of those aggregates in the lungs.

Keywords: solid lipid nanoparticles; transfection; "*in vitro*"; "*in vivo*"; non-viral vectors

1. INTRODUCTION

Naked plasmid DNA (pDNA) is a powerful tool for gene therapy compared to other non-viral and viral vectors because of its easier preparation, greater safety and stability and biochemical simplicity[1]. However, naked DNA is rapidly eliminated from the circulation after intravenous administration, due to the digestion by nucleases and to the hepatic uptake clearance, which occurs preferentially by non-parenchymal cells in a manner specific for polyanions[1,2,3]. Consequently, local injection is the preferred administration route, which seriously limits the therapeutic applications of these pharmaceuticals. To date the administration of naked pDNA has only provided systemic high level of gene expression "*in vivo*" by administrating pDNA with a large volume of saline at a high velocity into the tail vein of mice, the so-called hydrodynamics-based transfection procedure[4,5]. Therefore, the development of optimized pDNA delivery systems is necessary for its successful and conventional clinical use.

Gene delivery using cationic lipids has gained increasing interest as an alternative for viral vectors, due to a variety of functions that helps to introduce genes into cells. The

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1 Liu F, Shollenberger LM, Conwell CC, Yuan X, Huang L. Mechanism of naked DNA clearance after intravenous injection. J Gene Med. 2007; 9: 613-619.

2 Kawabata K, Takakura Y, Hashida M. The fate of plasmid DNA after intravenous-injection in mice - involvement of scavenger receptors in its hepatic-uptake. Pharm Res. 1995; 12: 825-830.

3 Yoshida M, Mahato RI, Kawabata K, Takakura Y, Hashida M. Disposition characteristics of plasmid DNA in the single-pass rat liver perfusion system. Pharm Res. 1996; 13: 599-603.

4 Abe S, Hanawa H, Hayashi M, Yoshida T, Komura S, Watanabe R, et al. Prevention of experimental autoimmune myocarditis by hydrodynamics-based naked plasmid DNA encoding CTLA4-Ig gene delivery. J Card Fail. 2005; 11: 557-564.

5 Chang WW, Su IJ, Lai MD, Chang WT, Huang W, Lei HY. The role of inducible nitric oxide synthase in a murine acute hepatitis B virus (HBV) infection model induced by hydrodynamics-based in vivo transfection of HBV-DNA. J Hepatol. 2003; 39: 834-842.

molecular architecture of cationic transfection lipids consists of a positive charged polar headgroup, and a hydrophobic moiety[6]. These lipids combined with a solid lipid form positively charged solid lipid nanoparticles (SLNs) which condense negatively charged DNA through electrostatic interactions. A cationic complex named *lipoplex* is formed, which protects DNA from nuclease degradation[7], and interacts with the negatively charged cell membranes leading to endocytosis[8]. Although formulations of cationic lipids are available commercially, e.g. Lipofectamine™ (Invitrogen, Carlsbad, CA, USA), Effectene™ (Qiagen, Valencia, CA, USA), Transfectam® (Promega, Madison, WI, USA), most of them are only useful for "*in vitro*" experimentation.

"*In vitro-in vivo*" correlation of nucleic acid carrier systems is difficult because of the troubles to mimic all the barriers the DNA delivery systems have to overcome in a living animal. Some features of the vectors regarding complex formation, size, superficial charge, nuclease protection, transfection activity and cell viability can be tested "*in vitro*", but parts of the optimization of these vectors require "*in vivo*" experimentation. In those cases the steps of the nucleic acid systemic delivery "*in vivo*" have to be considered: circulation within the bloodstream, tissue distribution, cell uptake and intracellular trafficking.

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6 Gascón AR, Pedraz JL. Cationic lipids as gene transfer agents: a patent review. Expert Opin Ther Pat. 2008; 18: 515-524.

7 del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles: Formulation factors affecting cell transfection capacity. Int J Pharm. 2007; 339: 261-268.

8 Elouahabi A, Ruysschaert JM. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol Ther. 2005; 11: 336-347.

Cationic non-viral vectors form aggregates easily with erythrocytes[9], activate the complement system[10] or are opsonized by plasma proteins[11], which reduces the circulation time and limits the access to the targeting tissue. To circumvent these problems various approaches have been recently introduced, including the development of new cationic lipids that are serum resistant[12], the stabilization of the vectors by DNA condensing agents, such as polyamines[13] or protamine sulphate[14], and the shielding of the positive surface charge by the steric stabilization with poly(ethylene glycol) (PEG) chains[15].

The aim of the present work was to show the capacity of SLNs to transfect "*in vivo*" after intravenous administration to mice. SLNs were complexed with the plasmid pCMS-EGFP that encodes the Enhanced Green Fluorescent Protein (EGFP) and the SLN-DNA

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9 Sakurai F, Nishioka T, Saito H, Baba T, Okuda A, Matsumoto O, et al. Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. Gene Ther. 2001; 8: 677-686.

10 Moghimi SM, Szebeni J. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog Lipid Res. 2003; 42: 463-478.

11 Owens DE, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm. 2006; 307: 93-102.

12 Lewis JG, Lin KY, Kothavale A, Flanagan WM, Matteucci MD, DePrince RB, et al. A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. P Natl Acad Sci USA. 1996; 93: 3176-3181.

13 Escriou V, Ciolina C, Lacroix F, Byk G, Scherman D, Wils P. Cationic lipid-mediated gene transfer: effect of serum on cellular uptake and intracellular fate of lipopolyamine/DNA complexes. Biochim Biophys Acta. 1998; 1368: 276-288.

14 Sorgi FL, Bhattacharya S, Huang L. Protamine sulfate enhances lipid-mediated gene transfer. Gene Ther. 1997; 4: 961-968.

15 Hong KL, Zheng WW, Baker A, Papahadjopoulos D. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery. FEBS Lett. 1997; 400: 233-237.

vectors were characterized in terms of size, superficial charge, nuclease protection, "*in vitro*" transfection activity and cell viability.

2. MATERIALS AND METHODS

2.1 Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). Antibiotic/antimycotic, Nile Red and Triton® X-100 were purchased from Sigma-Aldrich (Madrid, Spain). Tween-80 was provided by Vencaser (Bilbao, Spain) and dichloromethane by Panreac (Barcelona, Spain). 1,2-Dioleoyl-3-Trimethylammonium-Propane Chloride Salt (DOTAP) was acquired from Avanti Polar Lipids, Inc.

Plasmid pCMS-EGFP encoding the enhanced green fluorescent protein (EGFP) was purchased from BD Biosciences Clontech (Palo Alto, US) and amplified by Dro Biosystems S.L. (San Sebastián, Spain).

The materials employed for the electrophoresis on agarose gel were acquired from Bio-Rad (Madrid, Spain).

Cell culture reagents were purchased from LGC Promochem (Barcelona, Spain) and the normal goat serum (NGS) from Chemicon International Inc. (Temecula, CA, USA).

Female balb/c nude mice weighing 18-22 g (5 weeks of age) were purchased from the Harlam Interfauna Ibérica S.L (Barcelona, Spain).

Primary antibody (polyclonal anti-GFP, IgG fraction) and secondary antibody (Alexa Fluor®488 goat anti-rabbit IgG) were provided by Invitrogen (Barcelona, Spain).

BD Viaprobe kit was provided by BD Biosciences (Belgium). Fluoromount was purchased from SouthernBiotech (Coultek, España).

2.2. Production of solid lipid nanoparticles (SLNs)

The SLNs were produced by a solvent emulsification-evaporation technique[16]. Precirol® ATO 5 was dissolved in dichloromethane (5% w/v), and then emulsified in an aqueous phase containing DOTAP (0.4% w/v) and Tween-80 (0.1% w/v). The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) for 30 s at 50 W. The organic solvent was then removed by evaporation using a magnetic agitator for 45 min, followed by vacuum conditions for 15 min. An SLN suspension was formed upon precipitation of the Precirol® ATO 5 in the aqueous medium. Finally, the SLNs were washed by centrifugation (3000 rpm, 20 min, x 3) using Millipore (Madrid, Spain) Amicon® Ultra centrifugal filters (100,000 MWCO).

2.3. Preparation of SLN-DNA vectors

SLN-DNA vectors were obtained by mixing the pCMS-EGFP plasmid with an aqueous suspension of SLNs under agitation for 30 min. The SLN:DNA ratio, expressed as the ratio of DOTAP:DNA (w/w), was fixed at 5:1.

2.4. Size and zeta potential measurements

Sizes of SLNs and SLN-DNA vectors were determined by photon correlation spectroscopy (PCS). Zeta potentials were measured by laser Doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in 0.1 mM NaCl (aq.).

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16 Mehnert W, Mader K. Solid lipid nanoparticles - Production, characterization and applications. Adv Drug Deliv Rev. 2001; 47: 165-196.

2.5. DNase I protection study and SDS-induced release of DNA

DNase I was added to SLN-DNA vectors to a final concentration of 1 U DNase I/2.5 μg DNA, and the mixtures were then incubated at 37 ºC for 30 min. Afterwards, 2% SDS solution was added to the samples to a final concentration of 1% to release the DNA from the SLNs treated or non-treated with DNAse I. The samples were then subjected to electrophoresis on a 0.8% agarose gel (containing 1% ethidium bromide for visualization) for 30 min at 120 V. The bands were observed with a Vilber-Lourmat TFX-20M transilluminator. Images were captured using a Bio-Rad DigiDoc digital camera. The integrity of the DNA in each sample was compared with a control of untreated DNA.

2.6. "*In vitro*" transfection

"*In vitro*" assays were performed with the cell line Human Embryonic Kidney (HEK293) obtained from the American Type Culture Collection (ATCC). Cells were maintained in Eagle´s Minimal Essential Medium with Earle´s BSS and 2 mM L-glutamine (EMEM) supplemented with 10% heat-inactivated horse serum and 1% antibiotic-antimycotic. Cells were incubated at 37 ºC under 5% CO_2 atmosphere and subcultured every 2 to 3 days using trypsin-EDTA. For transfection, HEK293 cells were seeded on 24 well plates at a density of 150,000 cells per well and allowed to adhere overnight.

The SLN-DNA vectors were diluted in HBS (Hepes Buffer Saline) and added to the cell cultures. In all cases, 2.5 μg of DNA were added. The cells were incubated with the vectors at 37 ºC, and after 4 h, the medium containing the vectors in the wells was refreshed with 1 mL of complete medium. The cells were then allowed to grow for another 7 days.

At the end of the incubation period, the cells were washed once with 300 μL of PBS (Phosphate Buffered Saline) and were detached with 300 μL of 0.05% trypsin/EDTA. Then cells were centrifuged at 1500g and the supernatant was discarded. Cells were resuspended with PBS and directly introduced to a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, USA). For each sample 10,000 events were collected.

Transfection efficacy was quantified by measuring the fluorescence of EGFP at 525 nm (FL1). For cell viability measurements, the BD Via-Probe kit was employed. The reagent (5 μL) was added to each sample, and after 10 min of incubation, the fluorescence corresponding to dead cells was measured at 650 nm (FL3).

2.7. "*In vivo*" transfection

Animals were handled in accordance with the Principles of Laboratory Animal Care (http://www.history.nih.gov/laws). Mice were quarantined for approximately 1 week prior to the study. They were housed under standard conditions and had ad libitum access to water and standard laboratory rodent diet. The SLN-DNA vectors were injected in standard way into the tail vein in a volume of 100 μL (60 μg of plasmid). Controls were employed by administering free DNA and SLNs without plasmid in the same way and volume. 3 days and 7 days post-injection the mice were sacrificed and the livers, lungs and spleen were removed, quick frozen in liquid nitrogen in tissue freezing medium (Jung, Leica) and thin sectioned on a cryostat (Cryocut 3000, Leica). The treatment was administered to three mice in each group.

2.8. Immunolabelling of EGFP in tissue sections

Cryostat sections (7-10 μm) were fixed with 4% paraformaldehyde during 10 min at room temperature. Following washing in PBS, sections were blocked and permeabilized

in PBS 0.1M, 0.1% Triton® X-100 and 2% normal goat serum (NGS) for 1 h at room temperature. Then sections were incubated in primary antibody (polyclonal anti-GFP, IgG fraction) for 2 h at room temperature. Following adequate washing in PBS, sections were incubated in secondary antibody (AlexaFluor®488 goat anti-rabbit IgG) for 45 min at room temperature. Finally, sections were washed again in PBS and coverslipped with Fluoromount G.

Images of the immunolabelling sections were captured with an inverted microscopy equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon).

2.9. Statistical analysis

Results are reported as mean values (SD = standard deviation). Statistical analysis was performed with SPSS 14.0 (SPSS®, Chicago, IL, USA). Normal distribution of samples was assessed by the Shapiro–Wilk test, and homogeneity of variance, by the Levene test. The different formulations were compared with the Student´s t-test, whereby differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Characterization of SLNs and SLN-DNA vectors

Table I lists the particle size, zeta potential and polydispersity index of SLNs and SLN-DNA vectors. The size did not change ($p > 0.05$) after the addition of DNA to SLNs (approximately 280 nm), but the zeta potential of SLN-DNA vectors was reduced to +28 mV, as compared to +43 mV of the SLNs ($p < 0.01$). The polidispersity index was obtained from the size measurement, and it increased from 0.4 for the SLNs to 0.6 for the SLN-DNA vectors.

Table 1. Size, polydispersity index (P.I.) and zeta potential of SLNs and SLN-DNA vectors

	SLNs	SLN-DNA
Size (nm)	286 (45)	276 (61)
P.I.	0.4 (0.1)	0.6 (0.1)
Zeta potencial (mV)	+43 (4)	+28 (2)*

Mean values (SD = Standard Deviation), for n=6. * $p < 0.01$ respect to SLNs

3.2. "*In vitro*" resistance against DNase I and SDS-induced release of DNA

The agarose gel electrophoresis in Figure 1 shows the bands corresponding to the DNA released from SLN-DNA vectors treated with DNase I (lane 3) and from non-treated SLN-DNA vectors (lane 4). Lane 1 corresponds to non-treated free DNA, and lane 2 to DNase I-treated free DNA. The absence of bands in this lane indicates that DNA was totally digested by the enzyme. Whilst the non-treated free DNA shows only two bands: supercoilled (SC) and open circular (OC), the DNA released from SLN-DNA treated with DNase I (lane 3) shows a new band, corresponding to the lineal DNA (L), although the two lower bands (L and SC) are next to each other. The DNA released from non-treated vectors (lane 4) shows only the SC and OC bands; therefore, the L band in lane 3 is not due to changes in DNA conformation during the complexation with SLNs but, rather, to a partial degradation of DNA by DNase I. However, after the treatment with DNase I, the SC band still shows the greatest intensity.

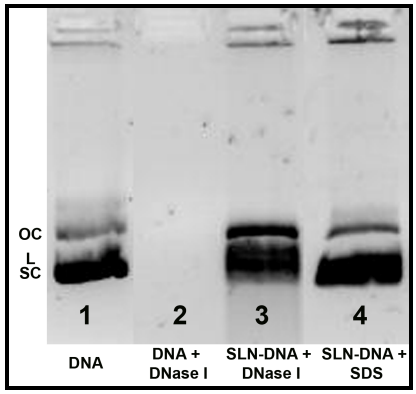

Figure 1. Protection of DNA from DNase I digestion, as visualized by agarose gel electrophoresis. OC: open circular form; L: lineal form; SC: supercoiled form.

3.3. "*In vitro*" transfection and cell viability

The percentages of HEK293 cells expressing EGFP were measured at different times from 1 h to 7 days after the addition of the vectors. As observed in Figure 2 (bars) green fluorescence was detected from 24 h, with 14% of the cells expressing EGFP, to 7 days, with 40% of EGFP positive cells.

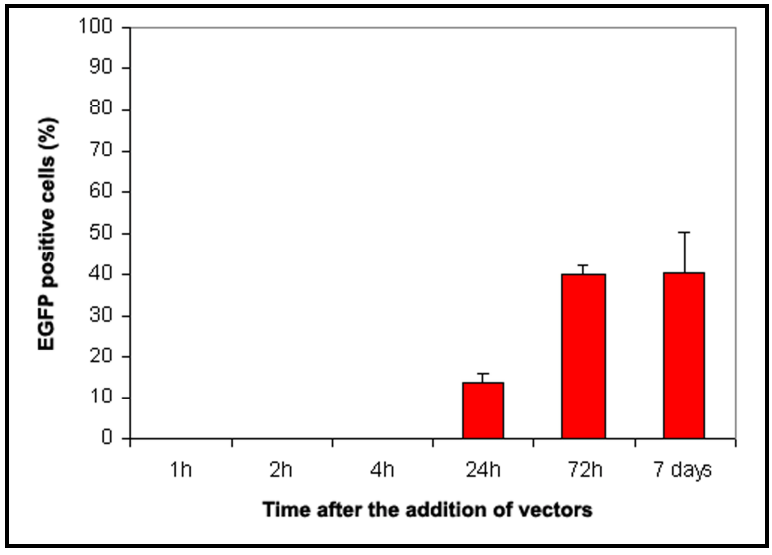

Figure 2. Transfection levels in HEK293 culture cells over time. The SLN:DNA ratio expressed as DOTAP:DNA ratio (w/w) was 5:1. Error bars represent SD (n=3).

In order to evaluate the effect of the formulations on cell viability, the percentage of dead cells was also determined by flow cytometry. The viability was near 75% over time. In the cultures of non-treated cells the same percentage of viable cells was observed ($p > 0.05$).

3.4. "*In vivo*" transfection

For the "*in vivo*" assays, mice were treated with free DNA or SLN-DNA vectors. Furthermore, in order to ensure that the observed green fluorescence was not an artifact of the immunolabelling, we subjected samples of mice treated with empty SLNs to the same procedure with the primary and the secondary antibodies, and no green fluorescence was detected.

The tissue sections of the mice treated with free DNA did not show fluorescence due to EGFP. However, the kidney and spleen sections from the mice treated with the SLN-DNA vectors did show that fluorescence (Figure 3). These sections were obtained from mice sacrificed 3 days and 7 days after the intravenous administration. From each tissue, 12 sections representing the whole organ were analyzed. On day 3, all sections showed EGFP, while on day 7 green fluorescence was only detected in some of the sections and the detection required a more thorough analysis. Therefore, on day 3 EGFP was more abundant and 7 days after the administration some protein still remained.

Lung sections were also studied, but no transfection was observed in this tissue.

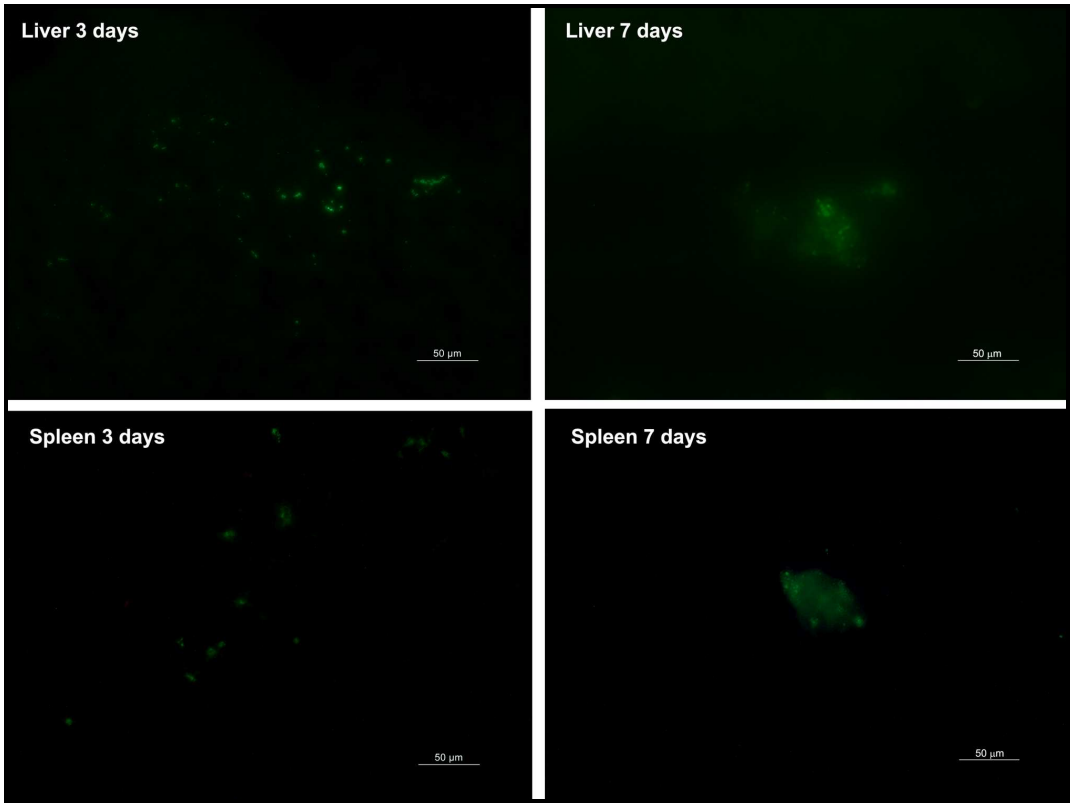

Figure 3. Images of the sections of liver and spleen after the immunolabelling of EGFP (green). Liver and spleen were removed from the sacrificed animals 3 and 7 days after the intravenous injection of SLN-DNA vectors (dose: 60 µg of pCMS-EGFP).

4. DISCUSSION

Systemic gene delivery by solid lipid nanoparticles (SLNs)-based non-viral vectors has not been reported to date, although they have demonstrated transfection capacity "*in vitro*"[7,17,18]. Prior to the "*in vivo*" application, an "*in vitro*" characterization of non-viral

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17 Bondi ML, Azzolina A, Craparo EF, Lampiasi N, Capuano G, Giammona G et al. Novel cationic solid-lipid nanoparticles as non-viral vectors for gene delivery. J Drug Targ. 2007; 15: 295-301.

18 Olbrich C, Bakowsky U, Lehr CM, Muller RH, Kneuer C. Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA. J Control Release. 2001; 77: 345-355.

vectors is necessary, in order to optimize some features that condition their behaviour in living animals, namely size, superficial charge or DNA protection.

For "*in vivo*" administration the size of the vectors is an important factor that may control the target organs because the structure of the blood capillary wall varies in different organs and tissues and in their state. In the present work, the SLN-DNA vectors showed a mean particle size of 276 nm, which is small enough to be intravenously administered. The endothelial cell layer of inflamed tissues or tumour tissues, for example, has gaps of 100-700 nm (depending on tumour type and localization)[19], which led the nanoparticles to escape from the vascular bed and migrate into those tissues. The particle size also influences the disposition of non-viral vectors in the lung. Mahato et al.[20] and Bragonzi et al.[21] have achieved higher transfection levels in the lung with liposomes of 400 nm or greater than with liposomes of 100 nm or smaller, due to the entrapment of the larger sized liposomes in the lung capillaries. Furthermore, after oral administration the size of the particles determines the uptake by Peyer's patches or the passage to blood[22].

The formation of positively charged nanoplexes by complexation of DNA with cationic carriers has some interesting features for gene therapy. Firstly, DNA degradation is

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19 Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP et al. Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. Cancer Res. 1995; 55: 3752-3756.

20 Mahato RI, Anwer K, Tagliaferri F, Meaney G, Leonard P, Wadhwa MS, et al. Biodistribution and gene expression of lipid/plasmid complexes after systemic administration. Hum Gene Ther. 1998; 9: 2083-2099.

21 Bragonzi A, Dina G, Villa A, Calori G, Biffi A, Bordignon C, et al. Biodistribution and transgene expression with nonviral cationic vector/DNA complexes in the lungs. Gene Ther. 2000; 7: 1753-1760.

22 Delie F. Evaluation of nano- and microparticle uptake by the gastrointestinal tract. Adv Drug Deliv Rev. 1998; 34: 221-233.

prevented. This aspect is usually studied "*in vitro*" by treating the vectors with DNase I. The gel electrophoresis in Figure 2 shows that SLNs partially protect the plasmid from this enzyme. Although the presence of nucleases in the cell culture is reduced, and protection of DNA is not critical, when the vectors are administered "*in vivo*" the genetic material is much more exposed to extra- and intracellular DNases which can damage DNA prior to entry into the nucleus.

The positive charge of SLN-DNA vectors is also advantageous because it facilitates the interaction with the negative charged cell surface and the cell entry. Elouahabi and Ruysschaert[8] have postulated that this positive charge could improve the entry in the cell facilitating the invagination of the cell plasma membrane and inducing the early steps of the endocytosis process, which has been previously identified[23,24,25] as a major mechanism of entry for non-viral vectors. However, positively charged vectors often interact with plasma components resulting in the formation of aggregates, which are mainly accumulated in the lungs because this is the first capillary vascular bed they encounter[26]. This is not only a limitation when tissues other than the lungs are targeted, but it is also a serious complication if it results in pulmonary embolism. Furthermore, foreign particles are usually opsonized by plasma proteins, as a natural process mediated by the innate immune system, leading to a rapid clearance from blood, due to the uptake

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23 del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, J.L. Pedraz. Solid lipid nanoparticles for retinal gene therapy: Transfection and intracellular trafficking in RPE cells. Int J Pharm. 2008; 360: 177-183.

24 Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther. 2005; 12: 468-474.

25 Rejman J, Conese M, Hoekstra D. Gene transfer by means of lipo- and polyplexes: Role of clathrin and caveolae-mediated endocytosis. J Liposome Res. 2006; 16: 237-247.

26 Kichler A. Gene transfer with modified polyethylenimines. J Gene Med. 2004; 6: S3-S10.

by reticuloendothelial system (RES). Another aspect to keep in mind is the activation of the complement system, which has been correlated to a high surface charge or to large complexes[10]. When the complement system is activated those complexes are removed by the mononuclear phagocyte system (MPS).

Various approaches have been used to overcome the interaction with blood constituents: new more serum-resistant lipids[12], neutral helper lipids[9], DNA-condensing agents, such as polyamines[13] or protamine sulphate[14], and the shielding of the positive charge of vectors by the attachment of water-soluble, neutral, flexible polymers, such as poly(ethyleneglycol) (PEG)[10,27,28]. The particles obtained with this last strategy are known as “stealth” particles. Enrichment of vectors with those molecules can be performed by physical adsorption, incorporation during the production of the carriers, or by covalent attachment to any reactive surface groups. In our vectors we have included Tween-80, which possesses PEG chains in its structure. On the one hand, those chains prevent each DNA molecule from binding more than one particle and, therefore, the formation of large aggregates is avoided[29]. On the other hand, the steric barrier neutralizes the excess of positive charges of the systems and reduces the interaction with blood components. However, the shielding strategy has a major disadvantage: the interaction with the target cells is usually reduced as consequence of the neutralization of positive charge.

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27 Harvie P, Wong Frances M, Bally Marcel B. Use of poly(ethyleneglycol)-lipid conjugates to regulate the surface attributes and transfection activity of lipid-DNA particles. J Pharm Sci. 2006; 89: 652-663.

28 Tranchant I, Thompson B, Nicolazzi C, Mignet N, Scherman D. Physicochemical optimisation of plasmid delivery by cationic lipids. J Gene Med. 2004; 6: S24-S35.

29 Liu F, Yang JP, Huang L, Liu D. Effect of non-ionic surfactants on the formation of DNA/emulsion complexes and emulsion-mediated gene transfer. Pharm Res. 1996; 13: 1642-1646.

The intravenous administration in mice of the SLNs composed by Precirol® ATO 5, DOTAP and Tween-80 led to transfection in hepatic tissue and spleen. However, EGFP was not detected in lungs. It is well-known that when cationic lipid-based systems are intravenously administered, approximately 80% of the dose accumulates in the lungs and substantial gene expression is achieved in that tissue[9,26,30]. As we have mentioned above, the accumulation in lungs is related to the formation of large aggregates when non-viral vectors interact with blood components[26]. In the present work the vectors had a positive charge of +28 mV and, although the PEG chains of Tween-80 did not totally neutralize the positive charges, the shielding effect seems to be enough to avoid in part the interaction with blood components. This is consistent with the "*in vitro*" transfection levels achieved in presence of serum (40% EGFP positive cells). The presence of Tween-80 in the formulations seems to reduce the interactions, with blood components avoiding the formation of aggregates and the accumulation into the lungs. Stealth particles are also supposed to be ignored by opsonines, although the clearance of PEG-coated particles by macrophages of the liver and the spleen (RES) has been reported, leading to a rapid hepatic and splenic deposition of a fraction of the dose[31,32,33,34,35,36].

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30 Eliyahu H, Servel N, Domb AJ, Barenholz Y. Lipoplex-induced hemagglutination: potential involvement in intravenous gene delivery. Gene Ther. 2002; 9: 850-858.

31 Allen TM, Hansen C, Martin F, Redemann C, Yauyoung A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim Biophys Acta. 1991; 1066: 29-36.

32 Laverman P, Brouwers AH, Dams ETM, Oyen WJG, Storm G, Van Rooijen N, et al. Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose. J Pharmacol Exp Ther. 2000; 293: 996-1001.

33 Laverman P, Boerman OC, Oyen WJG, Corstens FHM, Storm G. In vivo applications of PEG liposomes: Unexpected observations. Crit Rev Ther Drug. 2001; 18: 551-566.

This fact would explain the expression of EGFP observed in liver and spleen after systemic administration of our SLNs.

RES clearance can be useful if liver or spleen are the target tissue, for example, in vaccination (DNA vaccines), for the treatment of tumours in those organs or for the secretion into the bloodstream of lacking proteins in the organism. The spleen has been more frequently studied as targeted organ for DNA-based vaccines[37,38] as it is a secondary lymphoid organ involved in the initiation of immune responses, bringing together antigen and naive lymphocytes in organized microenviroments that support the antigen-specific clonal expansion of these cells and their differentiation into memory-effectors subsets. The targeting to the liver is not only useful for DNA vaccines[39], but also for the treatment of diseases such as cancer[40] or hepatitis B[41], as well as for the creation

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34 Laverman P, Carstens MG, Boerman OC, Dams ETM, Oyen WJG, Van Rooijen N, et al. Factors affecting the accelerated blood clearance of polyethylene glycol-liposomes upon repeated injection. J Pharmacol Exp Ther. 2001; 298: 607-612.

35 Utkhede DR, Tilcock CP. Studies upon the toxicity of polyethylene glycol coated lipid vesicles: Acute hemodynamic effects, pyrogenicity and complement activation. J Liposome Res. 1998; 8: 537-550.

36 Woodle MC, Matthay KK, Newman MS, Hidayat JE, Collins LR, Redemann C, et al. Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. Biochim Biophys Acta. 1992; 1105: 193-200.

37 Nakamura J, Fumoto S, Kawanami R, Kodama Y, Nishi J, Nakashima M, et al. Spleen-selective gene transfer following the administration of naked plasmid DNA onto the spleen surface in mice. Biol Pharm Bull. 30: 2007; 941-945.

38 Tupin E, Poirier B, Bureau MF, Khallou-Laschet J, Vranckx R, Caligiuri G et al. Non-viral gene transfer of murine spleen cells achieved by in vivo electroporation. Gene Ther. 2003; 10: 569-579.

39 Raska M, Moldoveanu Z, Novak J, Hel Z, Novak L, Bozja J, et al. Delivery of DNA HIV-1 vaccine to the liver induces high and long-lasting humoral immune responses. Vaccine. 2008; 26: 1541-1551.

40 Tang H, Tang XY, Liu M, Li X. Targeting alpha-fetoprotein represses the proliferation of hepatoma cells via regulation of the cell cycle. Clin Chim Acta. 2008; 394: 81-88.

of a depot organ, which produces large amounts of a therapeutic enzyme that is secreted to the bloodstream and recaptured by target organs[42].

The transfection studies "*in vitro*" and "*in vivo*" show that the EGFP expression was maintained at least 7 days. "*In vivo*" transfection in the liver and in the spleen progressively decreased over time, but "*in vitro*" the levels did not vary from 72h to 7 days. These differences could be due to the different type of transfected cells, and to the characteristics of the environment of transfected cells "*in vitro*" and "*in vivo*".

This work shows the capacity of SLNs to transfect "*in vivo*" by conventional intravenous administration in mice, which is more suitable than the hydrodynamics-based procedure. This latter procedure induces very high levels of transgene expression in the liver[43,44], but involves several risks for clinical use, due to rapid intravenous injection of an extraordinarily large volume of solution. Therefore, the hydrodynamic injection is nowadays used in laboratory animals as a tool to evaluate the therapeutic activity of certain genes[45], the analysis of regulatory functions of DNA sequences[46], the design of

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41 Zhang Y, Rong Qi X, Gao Y, Wei L, Maitani Y, Nagai T. Mechanisms of co-modified liver-targeting liposomes as gene delivery carriers based on cellular uptake and antigens inhibition effect. J Control Release. 2007; 117: 281-290.

42 Mango RL, Xu L, Sands MS, Vogler C, Seiler G, Schwarz T, et al. Neonatal retroviral vector-mediated hepatic gene therapy reduces bone, joint, and cartilage disease in mucopolysaccharidosis VII mice and dogs. Mol Genet Metab. 2004; 82: 4-19.

43 Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 1999; 6: 1258-1266.

44 Zhang GF, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther. 1999; 10: 1735-1737.

45 Liu D, Knapp JE. Hydrodynamics-based gene delivery. Curr Opin Mol Ther. 2008; 3: 192-197.

46 Kramer MG, Barajas M, Razquin N, Berraondo P, Rodrigo M, Wu C, et al. In vitro and in vivo comparative study of chimeric liver-specific promoters. Mol Ther. 2003; 7: 375-385.

RNA interference (RNAi) used to silence the expression of targeted genes[47] and the pharmacokinetics of gene medicines[48].

Our formulations showed transfection capacity when administered "*in vivo*" to mice, but further studies are needed in order to know if other organs can be transfected and to study other administration routes. Different strategies could be used in order to obtain more efficient systems with higher transfection levels and prolonged in time. On the one hand, the inclusion of targeting ligands on these formulations can be useful to direct the vectors to different organs, depending on the disease to be treated; e.g. RGD sequences for neurons or tumour endothelial cells, transferrin or folic acid for tumour cells, lactoferrin for brain, or sugars for hepatocytes. On the other hand, the incorporation in the vectors of molecules which have demonstrated improvement of transfection efficacy "*in vitro*", such as cell penetrating peptides (CPP)[49] or nuclear localization signals (NLS)[50], may result in more relevant benefits.

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47 Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med. 2003; 9: 347-351.

48 Kobayashi N, Nishikawa M, Takakura Y. The hydrodynamics-based procedure for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels. Adv Drug Deliv Rev. 2005; 57: 713-731.

49 del Pozo-Rodríguez A, Pujals S, Delgado D, Solinís MA, Gascón AR, Giralt E, et al. A proline-rich peptide improves cell transfection of solid lipid nanoparticle-based non-viral vectors. J Control Release. 2009; 133. 52-59.

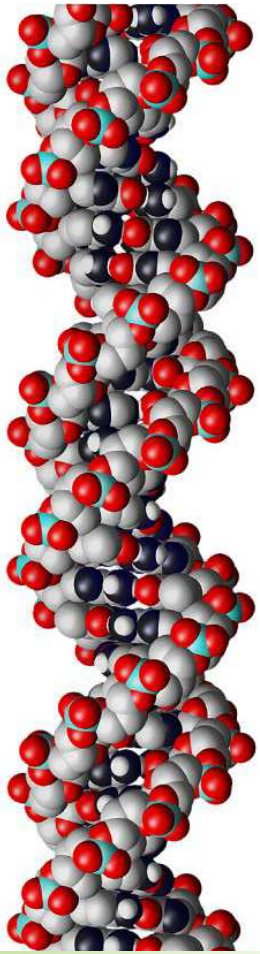
50 Ma H, Zhu J, Maronski M, Kotzbauer PT, Lee VMY, Dichter MA et al. Non-classical nuclear localization signal peptides for high efficiency lipofection of primary neurons and neuronal cell lines. Neuroscience. 2002; 112: 1-5.

5. CONCLUSIONS

On the basis of these results we can conclude that SLN-based vectors are able to transfect "*in vitro*" and "*in vivo*", inducing the expression of foreign proteins in the spleen and the liver, which is maintained for at least 7 days.

ACKNOWLEDGEMENTS

We would like to thank the Spanish Ministry of Education and Science for the research grant awarded to Ana del Pozo-Rodríguez (AP2003-4780). This work was supported by the Departamento de Educación, Universidades e Investigación del Gobierno Vasco (IT-407-07). Authors also want to thank Gontzal García, from the Neurosciences Department, for his assistance with the immunolabelling of EGFP in tissue sections.



DISCUSIÓN

Gracias a la descodificación del genoma humano, la terapia génica puede llegar a ser un arma terapéutica de uso habitual que permita curar enfermedades para las que actualmente sólo existe tratamiento sintomático.

Una terapia génica eficaz y segura requiere que el gen terapéutico llegue específicamente a las células diana y que penetre en el núcleo celular, de manera que se produzca la proteína correspondiente en cantidad suficiente para tratar el trastorno biológico objeto de la terapia. Este proceso se conoce como transferencia génica.

La transferencia génica se va a ver influenciada de manera importante por el tipo de sistema de administración empleado, condicionando la eficacia de transfección. Los vectores virales son capaces de transportar el ADN al núcleo de las células por sí mismos aprovechando su capacidad infectiva. Gracias a ello, con estos vectores se obtienen eficacias de transfección altas, aunque el riesgo de los virus de generar inmunogenicidad y oncogenicidad limita su utilización. Estos riesgos se evitan con el uso de vectores no virales; sin embargo, estos sistemas presentan más dificultades para transferir los genes al núcleo celular, reduciéndose de manera importante la transfección. Actualmente, numerosos grupos de investigación estamos centrados en el desarrollo de estrategias que permitan aumentar la eficacia de transfección de los sistemas no virales sin comprometer su seguridad.

El ADN plasmídico desnudo (pADN) es una potente herramienta para la terapia génica, debido a su fácil preparación, mayor seguridad y simplicidad bioquímica[1]. Sin embargo, su eficacia es baja debido a la degradación por enzimas nucleasas y a la rápida eliminación en el hígado, que en el caso de las moléculas aniónicas tiene lugar preferentemente a través de las células no parenquimales[1,2,3]. La formulación del pADN en un sistema de administración, como las nanopartículas sólidas lipídicas (SLNs), pretende evitar los inconvenientes del ADN desnudo.

La administración de material genético puede llevarse a cabo utilizando una vía local o una vía sistémica; la elección de una u otra vía de administración depende principalmente de la patología a tratar y del sistema de administración utilizado. La administración local da lugar a transfecciones mayores y más específicas; sin embargo, su aplicación se ve limitada a unos pocos órganos o tejidos. La administración sistémica se puede aplicar en un rango de órganos más amplio y es más sencilla; sin embargo, presenta menor especificidad y menor eficacia de transfección. Hasta el momento, la administración de pADN sólo ha permitido obtener niveles de expresión genética altos mediante su administración sistémica a través de la vena de la cola del ratón con un gran volumen salino a alta velocidad, lo que se conoce como administración hidrodinámica[4,5].

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1 Liu F, Shollenberger LM, Conwell C, Yuan X, Huang L. Mechanism of naked DNA clearance after intravenous injection. J Gene Med. 2007; 9: 613-619.

2 Kawabata K, Takakura Y, Hashida M. The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. Pharm Res. 1995; 12: 825-830.

3 Yoshida M, Mahato RI, Kawabata K, Takakura Y, Hashida M. Disposition characteristics of plasmid DNA in the single-pass rat liver perfusion system. Pharm Res. 1996; 13: 599-603.

4 Abe S, Hanawa H, Hayashi M, Yoshida T, Komura S, Watanabe R, et al. Prevention of experimental autoimmune myocarditis by hydrodynamics-based naked plasmid DNA encoding CTLA4-Ig gene delivery. J Card Fail. 2005; 11: 557-564.

Este tipo de administración conlleva ciertos riesgos debido a la rápida administración de un volumen extraordinariamente grande por vía intravenosa. Por ello, actualmente la administración hidrodinámica se utiliza únicamente en animales de laboratorio como herramienta para evaluar la actividad de ciertos genes[6], analizar las funciones regulatorias de secuencias de ADN[7], diseñar ARN de interferencia (ARNi)[8] empleados para silenciar la expresión de ciertos genes y estudiar la farmacocinética de genes terapéuticos[9]. Por lo tanto, para poder utilizar los pADN como medicamento de manera rutinaria en la clínica es necesario desarrollar y optimizar los sistemas de administración.

La administración de genes utilizando lípidos catiónicos ha adquirido gran interés como alternativa a los vectores virales debido a que, gracias a sus características, ayudan a introducir el ADN en el interior de las células. La arquitectura molecular de los lípidos catiónicos consiste en un grupo polar cargado positivamente y una parte hidrofóbica[10]. Estos lípidos, combinados con otro lípido sólido forman las nanopartículas sólidas lipídicas (SLNs) catiónicas, que condensan el ADN cargado negativamente mediante interacciones electrostáticas. De esta manera, se forma lo que se conoce como

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5 Chang WW, Su IJ, Lai MD, Chang WT, Huang W, Lei HY. The role of inducible nitric oxide synthase in a murine acute hepatitis B virus (HBV) infection model induced by hydrodynamics-based in vivo transfection of HBV-DNA. J Hepatol. 2003; 39: 834-842.

6 Liu D, Knapp JE. Hydrodynamics-based gene delivery. Curr Opin Mol Ther. 2008; 3: 192-197.

7 Kramer MG, Barajas M, Razquin N, Berraondo P, Rodrigo M, Wu C, et al. In vitro and in vivo comparative study of chimeric liver-specific promoters. Mol Ther. 2003; 7: 375-385.

8 Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med. 2003; 9: 347-351.

9 Kobayashi N, Nishikawa M, Takakura Y. The hydrodynamics-based procedure for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels. Adv Drug Deliv Rev. 2005; 57: 713-731.

10 Gascón AR, Pedraz JL. Cationic lipids as gene transfer agents: a patent review. Expert Opin Ther Pat. 2008; 18: 515-524.

lipoplex, capaz de proteger el ADN de la degradación por nucleasas[11], y de interaccionar con la membrana celular, cargada negativamente, dando lugar incluso al inicio de la endocitosis[12]. Aunque actualmente existen formulaciones basadas en lípidos catiónicos disponibles comercialmente, como por ejemplo Lipofectamina™ (Invitrogen, Carlbad, CA, USA), Effectene™ (Qiagen, Valencia, CA, USA) o Transfectam® (Promega, Madison, WI, USA), sólo son útiles para la experimentación "*in vitro*".

La correlación "*in vitro*"-"*in vivo*" de los sistemas de administración de ADN es difícil, debido a los problemas para simular todas las barreras que estos sistemas deben superar cuando se administran en un animal vivo. Algunas características de estos vectores, como la formación del complejo con el gen, el tamaño, la carga superficial, la protección frente a nucleasas, la eficacia de transfección o la toxicidad celular, pueden ser evaluadas "*in vitro*", pero parte del proceso de optimización requiere experimentación "*in vivo*", ya que deben ser tenidas en cuenta las barreras que estos vectores deben superar tras su administración a nivel sistémico: circulación en el torrente sanguíneo, distribución tisular, captación por las células y disposición intracelular.

Este trabajo recoge la caracterización y optimización de un sistema de administración de genes basado en SLNs. El plásmido utilizado ha sido el pCMS-EGFP, que codifica la proteína verde fluorescente (EGFP). Las formulaciones se caracterizaron determinándose el tamaño de partícula, la carga superficial y la capacidad de protección del ADN frente a nucleasas. Asimismo, se ha evaluado la eficacia de transfección y la

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11 del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles: formulation factors affecting cell transfection capacity. Int J Pharm. 2007; 339: 261-268.

12 Elouahabi A, Ruysschaert JM. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol Ther. 2005; 11: 336-347.

viabilidad celular "*in vitro*" en cultivos celulares, y la capacidad de transfección "*in vivo*" tras su administración intravenosa en ratones Balb/c. También se ha optimizado el proceso de liofilización y se ha llevado a cabo un estudio de estabilidad a corto y medio plazo de las formulaciones liofilizadas.

OPTIMIZACIÓN DE LAS SLNs COMO SISTEMAS NO VIRALES DE ADMINISTRACIÓN DE ADN

Las SLNs estudiadas se elaboraron mediante una técnica de emulsificación/evaporación del solvente[13]. Los componentes utilizados son el lípido sólido Precirol® ATO 5, el lípido catiónico DOTAP y el tensioactivo Tween-80. En la Figura 10 se recoge un esquema del proceso de elaboración.

13 Mehnert W, Mader K. Solid lipid nanoparticles - Production, characterization and applications. Adv Drug Deliv Rev. 2001; 47: 165-196.

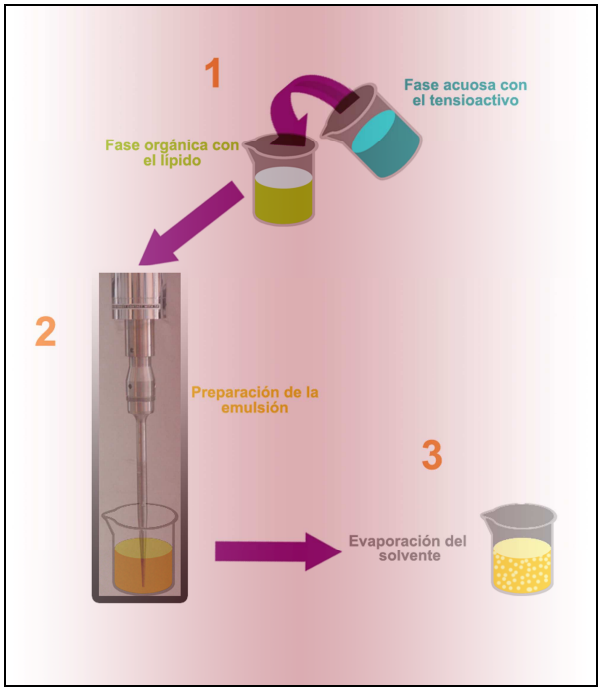

Figura 1. Esquema del proceso de elaboración de las SLNs mediante la técnica de emulsificación/evaporación del solvente.

El DOTAP es uno de los lípidos catiónicos más utilizado en terapia génica. Su presencia en las SLNs es necesaria debido a su actividad tensioactiva, que permite formar la emulsión inicial, y a su carga positiva, que proporciona a las nanopartículas carga neta positiva capaz de interaccionar electrostáticamente con la carga negativa del ADN. Para preparar las SLNs, la mínima proporción de DOTAP necesaria para obtener una emulsión estable fue del 1%. Con el fin de disminuir la proporción de DOTAP en las nanopartículas, para no comprometer la viabilidad celular, se incorporó un co-tensioactivo, el Tween-80. Este tensioactivo es uno de los más utilizados en la industria farmacéutica, y además presenta una serie de características que resultan interesantes para su uso en formulaciones destinadas a terapia génica, fundamentalmente debidas a la presencia de cadenas de poli(etilenglicol) (PEG) en su estructura. Diversos

autores[14,15,16,17] han demostrado que los tensioactivos no iónicos que presentan el grupo PEG en su estructura mejoraron la capacidad de transfección. Se probaron varias proporciones, siendo la proporción DOTAP 0,4% y Tween-80 0,1% la que proporcionó las eficacias de transfección más altas cuando se llevó a cabo el estudio en cultivos de células HEK293 (células humanas de riñón embrionario).

Para preparar los vectores SLN-ADN se optimizó la relación en peso DOTAP:ADN. Esta relación va a determinar el tamaño, la carga superficial y la protección frente a nucleasas de los vectores, factores que, a su vez, van a condicionar la capacidad de transfección. Las SLNs condensan el ADN y reducen su tamaño facilitando la movilidad en el interior de las células y la protección frente a las DNasas. Para conseguir unos niveles óptimos de transfección la relación DOTAP:ADN debe ser la adecuada para que se de un equilibrio entre el tamaño, la carga superficial positiva, la capacidad de liberación del plásmido antes de entrar en el núcleo y el grado de protección.

El tamaño de los vectores no virales va a condicionar la transfección. Sin embargo, existe cierta controversia al respecto, ya que diferentes autores han publicado resultados contradictorios. Prabha y col.[18] estudiaron la capacidad de transfección de partículas

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14 Kim TW, Kim YJ, Chung H, Kwon IC, Sung HC, Jeong SY. The role of non-ionic surfactants on cationic lipid mediated gene transfer. J Control Release. 2002; 82: 455-465.

15 Kim TW, Chung H, Kwon IC, Sung HC, Jeong SY. Optimization of lipid composition in cationic emulsion as *In vitro* and *In vivo* transfection agents. Pharm Res. 2001; 18: 54-60.

16 Liu F, Yang JP, Huang L, Liu D. Effect of non-ionic surfactants on the formation of DNA/emulsion complexes and emulsion-mediated gene transfer. Pharm Res. 1996; 13: 1642-1646.

17 Liu F, Yang JP, Huang L, Liu D. New cationic lipid formulations for gene transfer. Pharm Res. 1996; 13: 1856-1860.

18 Prabha S, Zhou W-Z, Panyam J, Labhasetwar V. Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. Int J Pharm. 2002; 244: 105-115.

poliméricas compuestas por el ácido poli-láctico co-glicólico (PLGA) en células COS-7 y HEK293. Con complejos de tamaño inferior a 100 nm obtuvieron un 27% más de transfección en las células COS-7 y un 4% en las células HEK293, en comparación con complejos de 298,2 nm. Almofti y col.[19], en cambio, demostraron que la transfección de las líneas celulares AH130 y SCB-3 mejoró a medida que aumentaba el tamaño de los vectores elaborados con el lípido catiónico DC-6-14 y DOPE, obteniendo los máximos niveles de transfección con tamaños superiores a 1 µm. En ambos estudios utilizaron el gen de la luciferasa. Por otro lado, Kawaura y col.[20] realizaron estudios de transfección del gen de la enzima CAT (cloranfenicol transferasa) con liposomas catiónicos compuestos por derivados catiónicos del colesterol y DOPE, en células NIH3T3. Los mayores niveles de transfección los obtuvieron con tamaños entre 400 nm y 1400 nm. Estos estudios demuestran que la influencia del tamaño en la transfección "*in vitro*" depende tanto de la composición del sistema de administración como de la línea celular. Por otro lado, hay que tener en cuenta que "*in vivo*" el tamaño va a condicionar el órgano al que se dirija el ADN una vez administrado, ya que va a afectar a su captación por los órganos y tejidos del organismo.

La carga superficial o potencial zeta de los vectores no virales es otro factor determinante en el proceso de transfección. La carga superficial positiva facilita la interacción con la superficie celular, cargada negativamente. Elouahabi y Russchaert[12] han postulado que esta carga positiva incluso podría mejorar la entrada en las células

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19 Almofti MR, Harashima H, Shinohara Y, Almofti A, Li W, Kiwada H. Lipoplex size determines lipofection efficiency with or without serum. Mol Membr Biol. 2003; 20: 35-43.

20 Kawaura C, Noguchi A, Tadahide F, Nakanishi M. Atomic force microscopy for studying transfection mediated by cationic liposomes with a cationic cholesterol derivative. FEBS Lett. 1998; 421: 69-72.

facilitando la invaginación de la membrana celular e induciendo los primeros pasos del proceso de endocitosis. Además, "*in vivo*" la carga superficial condiciona el tiempo de permanencia en la circulación sistémica, la distribución tisular y la captación celular.

Una característica importante de los sistemas de administración de ADN es su capacidad para protegerlo de componentes del medio, fundamentalmente de la digestión por nucleasas. La acción de estas enzimas sobre el ADN también afecta a la topología del mismo. El ADN puede presentar tres isoformas, súper-enrollada (SC), abierta (OC) y lineal (L), de las cuales la SC ha sido documentada como la de mayor actividad biológica[21,22]. Cuando el ADN es digerido por desoxirribonucleasas (DNasas), estas enzimas en primer lugar hacen que la forma SC pase a la forma OC, para sucesivamente romper la forma OC en la forma L[23].

En este trabajo se estudiaron distintas relaciones DOTAP:ADN que variaron entre 15:1 y 1:1. Sólo cuando los vectores se elaboraron con proporciones entre 15:1 y 4:1 las nanopartículas fueron capaces de unir todo el ADN; además, se observó una disminución en la capacidad de condensación del plásmido a medida que la relación DOTAP:ADN disminuía. Otros autores han obtenido resultados similares a los nuestros[24,25,26]. La menor condensación del ADN se tradujo en un aumento del tamaño

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21 Middaugh CR, Evans RK, Montgomery DL, Casimiro DR. Analysis of plasmid DNA from a pharmaceutical perspective. J Pharm Sci. 1998; 87:130-146.

22 Remaut K, Sanders NN, Fayazpour F, Demeester J, De Smedt SC. Influence of plasmid DNA topology on the transfection properties of dotap/dope lipoplexes. J Control Release. 2006; 115: 335-343.

23 Sanders NN, De Smedt SC, Demeester J. En: McGrath BM, Walsh G, editors. Therapeutic Enzyms, Taylor & Fracis Group, Boca Raton; 2006. p. 97-116.

24 Mahato RI, Kawabata K, Nomura T, Takakura Y, Hashida M. Physicochemical and pharmacokinetic characteristics of plasmid DNA cationic liposome complexes. J Pharm Sci. 1995; 84: 1267-1271.

de los vectores, una reducción de la carga positiva, que alcanzó incluso valores negativos a partir de la relación 3:1 (Figura 11), y una disminución de la protección frente a la enzima DNasa I, debido a que el ADN está más expuesto.

DOTAP:DNA (w/w)	Tamaño (nm)	Potencial zeta (mV)
SLNs	339.1 (12.4)	+42.9 (1.3)
15:1	258.5 (23.1)	+40.6 (5.6)
10:1	216.7 (44.2)	+46.6 (4.4)
7:1	260.1 (18.7)	+38.9 (7.1)
6:1	255.9 (61.7)	+35.3 (2.6)
5:1	249.2 (25.3)	+36.5 (2.8)
4:1	240.4 (10.9)	+32.4 (0.9)
3:1	> 1 µm	-11.9 (5.2)
2.5:1	> 1 µm	-23.7 (2.9)
1:1	> 1 µm	-37.1 (1.9)

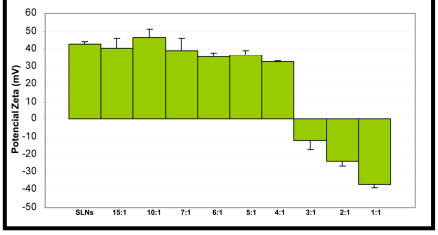

Figura 2. La gráfica inferior representa los cambios en el potencial zeta en función de la proporción DOTAP:ADN utilizada para elaborar los vectores SLN-ADN. La tabla superior recoge el tamaño y potencial zeta de los vectores SLN-ADN preparados con las distintas proporciones DOTAP:ADN; media (D.E.), (n=3).

La capacidad de proteger el ADN sólo se mantuvo cuando los vectores se elaboraron con relaciones DOTAP:ADN 4:1 o superiores. Como se aprecia en la Figura 12, a medida que la relación DOTAP:ADN fue disminuyendo la protección frente a la enzima DNasa I también se redujo, pero la banda correspondiente a la forma SC, la de mayor actividad biológica, se mantuvo.

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25 Ferrari ME, Nguyen CM, Zelphati O, Tsai YL, Felgner PL. Analytical methods for the characterization of cationic lipid nucleic acid complexes. Hum Gene Ther. 1998; 9: 341-351.

26 Faneca H, Simoes S, de Lima MCP. Evaluation of lipid-based reagents to mediate intracellular gene delivery. Biochim Biophys Acta. 2002; 1567: 23-33.

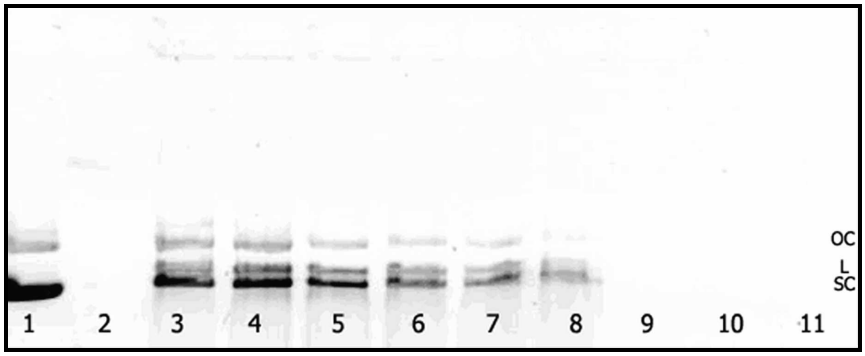

Figura 3. Electroforesis en gel de agarosa correspondiente al ensayo de protección del ADN frente a la enzima DNasa I con las diferentes proporciones SLN:ADN. Línea 1: ADN libre; línea 2: ADN libre tratado con DNasa I; línea 3: 15:1; línea 4: 10:1; línea 5: 7:1; línea 6: 6:1; línea 7: 5:1; línea 8: 4:1; línea 9: 3:1; línea 10: 2:1; línea 11: 1:1. OC: ADN abierto, L: ADN lineal, SC: ADN superenrollado.

Teniendo en cuenta los resultados anteriores, en el estudio de la capacidad de transfección "*in vitro*" se utilizaron las formulaciones elaboradas con una relación DOTAP:ADN entre 15:1 y 4:1 (Figura 13), que son aquellas capaces de unir el ADN, manteniendo la carga superficial positiva y protegiendo el plásmido de la degradación enzimática. Las formulaciones elaboradas con las relaciones DOTAP:ADN 15:1 y 10:1 dieron lugar a niveles de transfección (aproximadamente 10% de células transfectadas) menores que las elaboradas con las proporciones 7:1, 5:1 y 4:1 (aproximadamente 15% de células transfectadas). Estas diferencias pueden ser debidas al distinto grado de condensación del ADN. Si la condensación es excesiva, la liberación del ADN de los vectores puede verse dificultada limitando la transfección. De hecho, Sakurai y col.[27] consideran la liberación del ADN como uno de los pasos cruciales a la hora de determinar la proporción óptima lípido catiónico:ADN. Observando la Figura 3, la mayor

27 Sakurai F, Inoue R, Nishino Y, Okuda A, Matsumoto O, Taga T, et al. Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression. J Control Release. 2000; 66: 255–269.

intensidad de las bandas correspondientes a las proporciones DOTAP:ADN 15:1 y 10:1 indica una mayor capacidad de condensación, ya que el ADN está más protegido que en aquellas elaboradas con las relaciones 7:1, 5:1 y 4:1, con las que las bandas de ADN disminuyen considerablemente de intensidad tras el tratamiento con la enzima DNasa I.

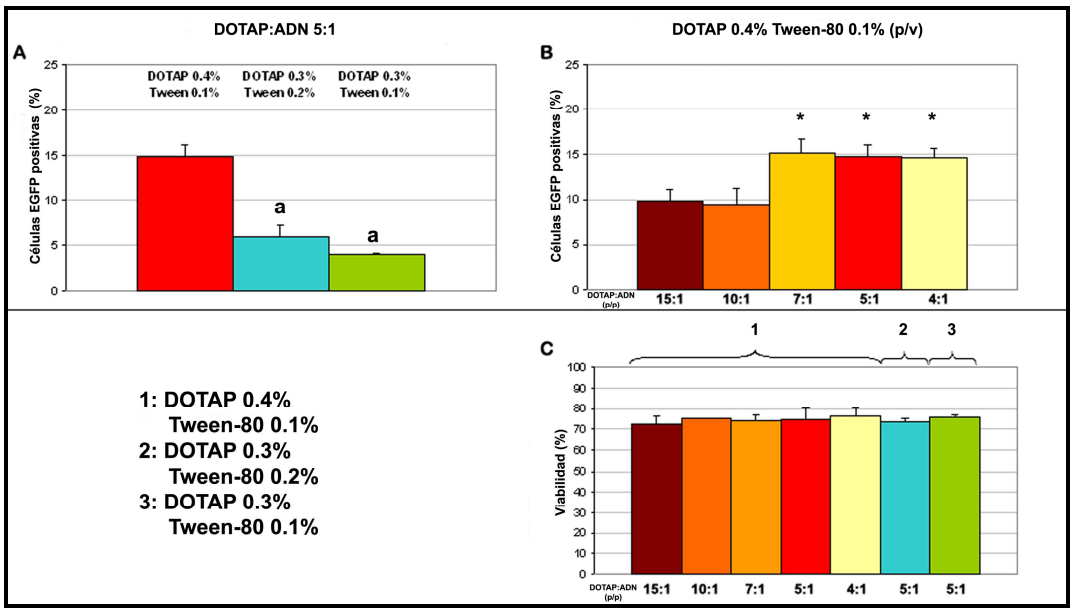

Figura 4. Niveles de transfección y viabilidad celular obtenidos con las distintas formulaciones estudiadas. (A) Influencia de la composición de las SLNs en la eficacia de transfección. (B) Influencia de la proporción DOTAP:ADN en la eficacia de transfección. (C) Viabilidad celular después del tratamiento con las diferentes formulaciones. $^{a}p < 0.05$ respecto a la formulación compuesta por DOTAP 0.4% y Tween-80 0.1%. (Barras de error = D.E., n=3) $^{*}p < 0.05$ respecto a las proporciones DOTAP:ADN 15:1 y 10:1.

La formulación seleccionada para llevar a cabo los siguientes estudios fue la elaborada con la relación DOTAP:ADN 5:1, ya que tiene menor cantidad de DOTAP que la formulación elaborada con la relación 7:1, lo cual deber ser tenido en cuenta porque se ha documentado la aparición de reacciones inflamatorias tras la administración

repetida de lípidos catiónicos[28]. Además la capacidad de protección de la formulación elaborada con la relación 5:1 es considerablemente mayor que la de la relación 4:1. Los vectores elaborados con la relación 5:1 presentaron un tamaño de partícula de 250 nm, una carga superficial de +36 mV, demostraron ser capaces de proteger el ADN frente a la DNasa y no disminuyeron la viabilidad celular respecto a las células no tratadas con los vectores.

INTERNALIZACIÓN Y DISPOSICIÓN INTRACELULAR DE LAS SLNs

Una vez caracterizados los vectores y conocida su capacidad de transfección "*in vitro*", el siguiente paso consistió en estudiar su mecanismo de entrada y disposición intracelular, lo que nos permite conocer los pasos limitantes en el proceso de transfección celular y buscar estrategias para mejorar la eficacia de transfección. Para ello, se utilizaron dos líneas celulares: HEK293 y ARPE-19 (células humanas del epitelio pigmentario de retina). La línea celular HEK293 es habitualmente utilizada en estudios de transfección "*in vitro*" con vectores no virales, y sus condiciones de cultivo y manipulación están optimizadas. Además, es una de las líneas celulares que mejor se transfecta por lo que estas células suponen un buen modelo para estudiar nuevos sistemas de administración de ADN. Las células del epitelio pigmentario de retina (RPE) se utilizan frecuentemente "*in vitro*" como herramienta para evaluar nuevas estrategias en la terapia génica para el tratamiento de las enfermedades de retina[29,30,31,32,33]. Las

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28 Han S, Mahato RI, Sung YK, Kim SW. Development of biomaterials for gene therapy. Mol Ther. 2000; 2: 302-317.

29 Chaum E, Hatton MP, Stein G. Polyplex-mediated gene transfer into human retinal pigment epithelial cells in vitro. J Cell Biochem. 2000; 76: 153–160.

SLNs fueron capaces de transfectar ambas líneas celulares, aunque los niveles de transfección en las células ARPE-19 fueron menores que en las HEK293 (2.5% frente al 14% de células EGFP-positivas, respectivamente)[34]. Además, en las células HEK293 la expresión de proteína se detectó a partir de las 24 h, mientras que en las células de retina no se observó hasta las 72 h. Este retraso en la producción de la proteína, así como la menor transfección obtenida, pueden ser debidos a las distintas barreras que el plásmido tienen que superar para llegar al núcleo y que se resumen en la Figura 14: la velocidad de entrada, el mecanismo de entrada y el paso al núcleo. La facilidad para que el ADN supere estas barreras va a depender del sistema de administración y de la línea celular.

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30 Doi K, Hargitai J, Kong J, Tsang SH, Wheatley M, Chang S, et al. Lentiviral transduction of green fluorescent protein in retinal epithelium: evidence of rejection. Vis Res. 2002; 42: 551–558.

31 Pitkanen L, Pelkonen J, Ruponen M, Ronkko S, Urtti A. Neural retina limits the nonviral gene transfer to retinal pigment epithelium in an in vitro bovine eye model. AAPS J. 2004; 6.

32 Bejjani R, Benezra D, Cohen H, Rieger J, Andrieu C, Jeanny JC, et al. Nanoparticles for gene delivery to retinal pigment epithelial cells. Mol Vis. 2005; 11: 124–132.

33 Mannisto M, Ronkko S, Matto M, Honkakoski P, Hyttinen M, Pelkonen J, et al. The role of cell cycle on polyplex-mediated gene transfer into a retinal pigment epithelial cell line. J Gene Med. 2005; 7: 466–476.

34 del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles for retinal gene therapy: transfection and intracellular trafficking in RPE cells. Int J Pharm. 2008; 360: 177-183.

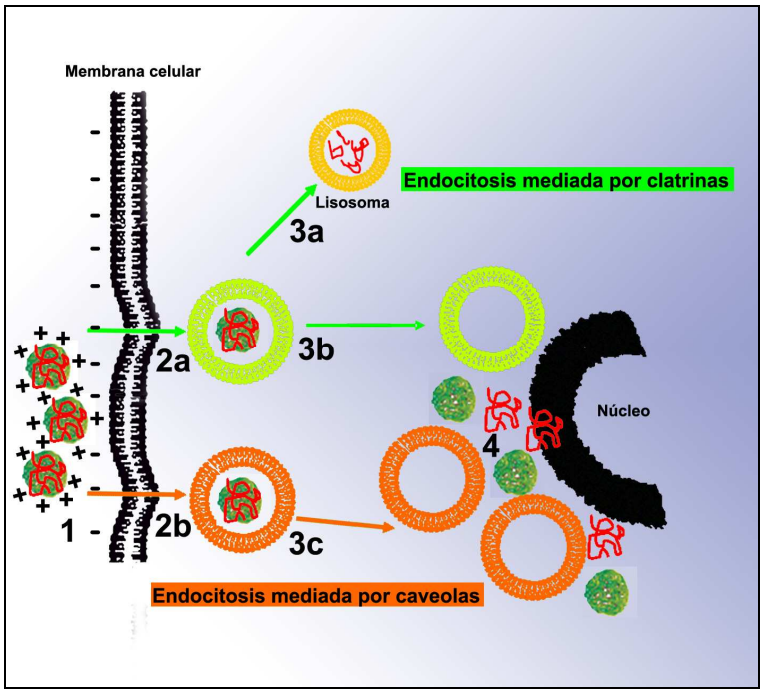

Figura 5. Esquema de las barreras que debe superar el ADN para llegar al núcleo. En primer lugar debe unirse a la membrana celular (1) para comenzar el proceso de entrada. La entrada se lleva a cabo principalmente mediante procesos de endocitosis (2). Si la endocitosis es mediada por clatrinas (2a) da lugar a una ruta intracelular en la que los endosomas se funden con lisosomas que tienden a degradar su contenido (3a); en este caso, es importante una salida rápida de los endosomas (3b). Si la endocitosis es mediada por caveolas (2b) se evita esa ruta lisosomal y por tanto la degradación de los vectores (3c). Una vez que el ADN consigue salir de los endosomas, el siguiente paso consiste en atravesar la membrana nuclear (4).

Con el objetivo de conocer las causas del retraso y la menor cantidad de producción de la proteína EGFP en las células de retina, en primer lugar estudiamos la entrada de los vectores en las dos líneas celulares (HEK293 y ARPE-19), utilizando para ello vectores SLN-ADN marcados con Nile Red, que emite fluorescencia roja. En los cultivos de células HEK293 el porcentaje de células que captaron vectores SLN-ADN se mantuvo entre el 50 y 60% (Figura 15) durante todo el tiempo del estudio (desde las 0.5 h hasta las 72 h). En los cultivos celulares de ARPE-19, el porcentaje de células que captaron vectores fue del 10-20% al inicio del estudio, aumentando hasta el 58% al final del

mismo (72 h), siendo este un nivel similar al observado en las células HEK293. Por lo tanto, la entrada de los vectores SLN-ADN en las células de retina es más lenta que la entrada en las células HEK293, pudiendo ser ésta la causa del retraso en la producción de la proteína EGFP.

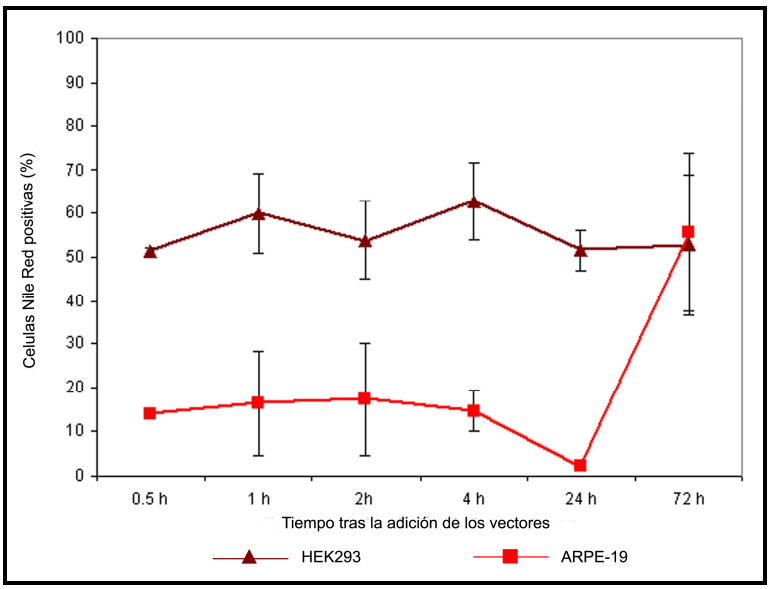

Figura 6. Captación de los vectores SLN-ADN marcados con Nile Red, por parte de las células HEK293 y ARPE-19 a diferentes tiempos (0.5 h – 72 h) tras la adición de los vectores. (Barras de error = D.E., n=3).

Además de la capacidad para captar partículas, el mecanismo de entrada es importante en el proceso de transfección, ya que va a condicionar la disposición intracelular de los vectores. La endocitosis se ha postulado como el principal mecanismo de entrada de los vectores no virales, describiéndose múltiples mecanismos de endocitosis: fagocitosis, pinocitosis, endocitosis mediada por clatrinas o mediada por caveolas. La contribución relativa de cada mecanismo a la internalización de los sistemas no virales ha sido poco estudiada, pero se sabe que la entrada a través de clatrinas da lugar a una ruta intracelular en la que los endosomas se funden con

lisosomas que tienden a degradar su contenido, mientras que la endocitosis mediada por caveolas evita esa ruta lisosomal y por tanto la degradación de los vectores. En consecuencia, en el caso de la endocitosis mediada por clatrinas es esencial una salida más rápida del ADN del compartimento endosomal, antes de que se produzca la degradación lisosomal.

Para conocer la vía de endocitosis utilizada y el comportamiento intracelular de los sistemas SLN-ADN en las células HEK293 y ARPE-19, se llevó a cabo un estudio con microscopía confocal, marcando las SLNs con Nile Red y estudiando su co-localización con marcadores de la entrada a través de clatrinas (transferrina) o caveolas (toxina del cólera). Aunque parece que la endocitosis mediada por clatrinas es la principal vía de entrada de los sistemas formulados con lípidos catiónicos, y específicamente de aquellos compuestos por DOTAP[35,36], nuestros resultados mostraron que en las células HEK293 los vectores SLN-ADN entran principalmente a través de caveolas, mientras en las ARPE-19 lo hacen a través de clatrinas[34]. Además, cuando se estudió la disposición de los vectores marcados a nivel intracelular, se apreció una importante diferencia (Figura 16): en las células HEK293 se encontraban distribuidos homogéneamente por todo el citoplasma, pero en las células de retina, los vectores se encontraban localizados en la zona correspondiente al retículo endoplásmico rugoso y al aparato de Golgi, donde son sintetizados los lisosomas. Estos resultados demuestran que la menor transfección

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35 Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther. 2005; 12: 468–474.

36 Rejman J, Conese M, Hoekstra D. Gene transfer by means of lipo- and polyplexes: role of clathrin and caveolae-mediated endocytosis. J Liposome Res. 2006; 16: 237–247.

obtenida en las células ARPE-19 puede deberse a la mayor entrada a través de clatrinas en esta línea celular.

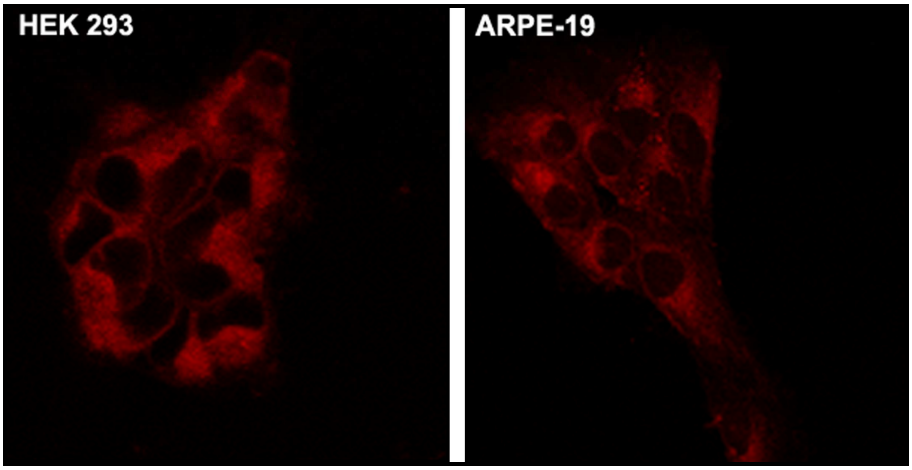

Figura 7. Diferencias en la disposición intracelular de los vectores en las células HEK293 y ARPE-19. Las imágenes se tomaron mediante microscopía confocal 2 horas después de añadir a los cultivos celulares SLNs marcadas con Nile Red (fluorescencia roja).

El último paso limitante en el proceso de transfección es la entrada del ADN al núcleo. En general este paso es difícil ya que la membrana nuclear es una barrera selectiva que evita la entrada de moléculas mayores de 40 kDa, tamaño que los plásmidos superan. Las moléculas de mayor tamaño pueden superar esa barrera de dos maneras: aprovechando la rotura de la membrana nuclear durante la mitosis o a través de los complejos de poro nuclear (NPC), lo que requiere señales de localización nuclear (NLS). Con nuestras formulaciones, el ADN debe entrar durante la mitosis, cuando la membrana nuclear desaparece temporalmente. Las células HEK293 se dividen más rápidamente[37] que las ARPE-19, por lo que la entrada al núcleo será más difícil en estas últimas. De

37 Thomas P, Smart TG. HEK293 cell line: a vehicle for the expression of recombinant proteins. J Pharmacol Toxicol Methods. 2005; 51: 187–200.

hecho, las células de retina se caracterizan por una lenta velocidad de división, que hace que la renovación de los constituyentes celulares constituya un fenómeno metabólico de gran importancia para mantener el volumen celular constante[38]. La importancia de la velocidad de división en el proceso de transfección mediado por lípidos catiónicos ya ha sido demostrada[39,40]. Las imágenes tomadas mediante microscopía de fluorescencia de ambas líneas celulares tratadas con vectores que portaban ADN marcado con monoazida de etidio (EMA), mostraron que en las células ARPE-19, al contrario que en las HEK293, la cantidad de ADN en el citoplasma fue mayor a las 72 h que a las 24 h, encontrándose la mayoría cerca de los núcleos celulares pero no dentro. Esta diferencia se debe, por un lado, a que los vectores, y por tanto el plásmido, entran más tarde en las células de retina, siendo la entrada máxima a las 72 h, y por otro a la división más lenta de las células ARPE-19, que dificulta la entrada del ADN al núcleo, haciendo que éste permanezca más tiempo en el citoplasma. Por lo tanto, el retraso observado en la expresión de la EGFP en las células de retina no es sólo debida a un retraso en la entrada de los vectores, sino también a la velocidad de división más lenta. Esto nos llevó a pensar que quizá en esta línea celular los niveles máximos de proteína se alcanzarían más tarde. Sin embargo, los niveles de proteína medidos a los 7 días disminuyeron. Este hecho probablemente se deba al mayor tiempo de permanencia del ADN libre en el citoplasma, expuesto, por tanto, a una mayor degradación.

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38 Runggerbrandle E, Englert U, Leuenberger PM. Exocytic clearing of degraded membrane material from pigment epithelial-cells in frog retina. Invest Ophth Vis Sci. 1987; 28: 2026-2037.

39 Mortimer I, Tam P, MacLachlan I, Graham RW, Saravolac EG, Joshi PB. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. Gene Ther. 1999; 6: 403–411.

40 Tseng WC, Haselton FR, Giorgio TD. Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. Biochim Biophys Acta. 1999; 1445: 53–64.

Nuestros resultados demuestran que el estudio del comportamiento intracelular de los sistemas de administración de ADN resulta muy útil para diseñar y optimizar formulaciones que tengan en cuenta las características específicas y las limitaciones de cada tipo de célula. En el caso de la línea celular ARPE-19 se pueden plantear varias estrategias para favorecer la transfección. Por un lado, se pueden incorporar sustancias que incrementen el paso de la membrana celular, como los péptidos de penetración celular (CPP)[41]. Teniendo en cuenta que el mecanismo de endocitosis en esta línea celular es mayoritariamente mediado por clatrinas, otras posibilidades serían adicionar sustancias que favorezcan el escape endosomal, como los co-lípidos DOPE y colesterol[42], antes de que los endosomas se fundan con lisosomas, o favorecer la entrada a través de caveolas[43,44]. Finalmente, también se puede recurrir a la incorporación de señales de localización nuclear (NLS) en los vectores SLN-ADN para favorecer la entrada al núcleo, disminuyendo el tiempo de permanencia en el citoplasma y, por tanto, su degradación.

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41 Gupta B, Levchenko TS, Torchilin VP. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. Adv Drug Deliv Rev. 2005; 57: 637-651.

42 Karmali PP, Chaudhuri A. Cationic Liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises. Med Res Rev. 2007; 27: 696–722.

43 Foerg C, Ziegler U, Fernandez-Carneado J, Giralt E, Rennert R, Beck-Sickinger AG, et al. Decoding the entry of two novel cell-penetrating peptides in HeLa cells: lipid raft-mediated endocytosis and endosomal escape. Biochemistry. 2005; 44: 72–81.

44 Pujals S, Fernandez-Carneado J, Ludevid D, Giralt E. D-SAP: a new, noncytotoxic and fully protease resistant cell-penetrating peptide. ChemMedChem. 2008; 3: 296–301

INCORPORACIÓN DEL PÉPTIDO DE PENETRACIÓN CELULAR *SAP* A LAS FORMULACIONES

El uso de péptidos capaces de atravesar las membranas celulares, los péptidos de penetración celular (CPP), es una nueva estrategia muy utilizada en terapia génica. Muchos de estos péptidos contienen aminoácidos cargados positivamente y son hidrofóbicos, normalmente debido a una secuencia anfipática[45]. Dentro de este grupo, el péptido SAP, cuya secuencia es $(VRLPPP)_3$ (compuesto por tres unidades repetidas de VRLPPP; V=Val, R=Arg, L=Leu, P=Pro), presenta ciertas ventajas sobre otros CPP debido a su origen no viral, anfipaticidad, solubilidad en agua, y el hecho de no ser citotóxico ni siquiera a concentraciones altas[46].

En este trabajo se han combinado SLNs y el péptido SAP con la idea de facilitar la entrada y la consiguiente disposición intracelular en las células de retina[47]. Para elaborar los vectores primero se unió el SAP al ADN en una relación en peso de 50:1 o superior. Posteriormente, los complejos SAP-ADN se pusieron en contacto con las SLNs para formar los vectores finales SAP-ADN-SLN mediante interacciones electrostáticas.

En primer lugar, se estudió la influencia del péptido en el tamaño y la carga final de los vectores, ya que pueden afectar a la eficacia de transfección. La presencia de SAP en las formulaciones no indujo cambios en la carga superficial, pero el tamaño sí se vio

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45 Khalil IA, Kogure K, Futaki S, Harashima H. Octaarginine-modified liposomes: Enhanced cellular uptake and controlled intracellular trafficking. Int J Pharm. 2008; 354: 39-48.

46 Fernandez-Carneado J, Kogan MJ, Castel S, Giralt E. Potential peptide carriers: Amphipathic proline-rich peptides derived from the n-terminal domain of gamma-zein. Angew Chem Int Edit. 2004; 43: 1811-1814.

47 del Pozo-Rodríguez A, Pujals S, Delgado D, Solinís MA, Gascón AR, Giralt E, et al. A proline-rich peptide improves cell transfection of solid lipid nanoparticle-based non-viral vectors. J Control Release. 2009; 133: 52-59.

reducido con algunas relaciones SAP:ADN, aunque la disminución fue inferior a los 100 nm. La adición del péptido SAP a las formulaciones no redujo su capacidad de protección frente a DNasas.

La incorporación del péptido en los vectores produjo un incremento en el nivel de transfección en las dos líneas celulares estudiadas, HEK293 y ARPE-19 (Figura 17). En las células HEK293, el aumento en la transfección se produjo con las tres proporciones SAP:ADN utilizadas (50:1, 100:1 y 200:1), pero en las células de retina sólo con la proporción 100:1. La viabilidad celular no se vio afectada por la presencia del SAP en las formulaciones.

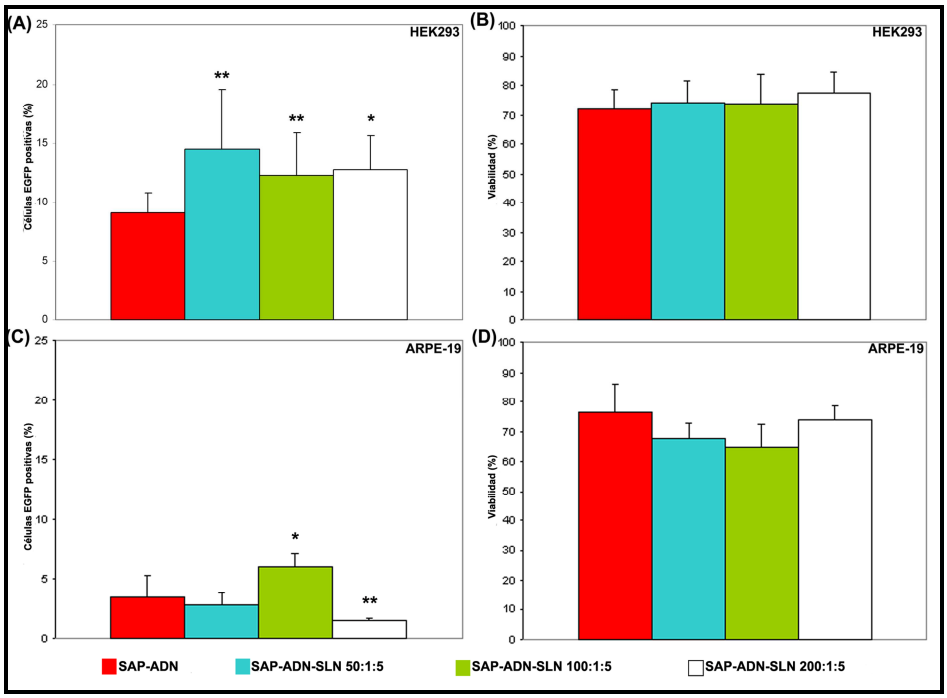

Figura 8. Transfeccion y viabilidad celular obtenida en cultivos de células HEK293 (A y B) y ARPE-19 (C y D) con las distintas proporciones SAP:ADN. La proporción SLN:ADN fue 5:1 en todos los casos. (Barras de error = D.E., n=3) * $p < 0.01$ respecto a la formulación SLN-ADN; ** $p < 0.05$ respecto a la formulación SLN-ADN.

Para conocer el mecanismo por el cual el péptido da lugar a un aumento de los niveles de transfección, llevamos a cabo estudios de la captación celular y el mecanismo de entrada de los vectores en ambas líneas celulares, y observamos que en el caso de las células HEK293, el aumento en la transfección se justifica por una mayor entrada de los vectores en las células, pero en las células de retina la captación fue similar en presencia o no del péptido. Por lo tanto, en las células ARPE-19 el péptido actúa mediante otro mecanismo para favorecer la transfección. Estudios previos han mostrado que en las células HeLa, el SAP es internalizado a través de un proceso de endocitosis mediado por caveolas[43,44]. Nosotros observamos, mediante estudios cualitativos y cuantitativos, que en las células ARPE-19 la incorporación del SAP a nuestros vectores favorece la endocitosis mediada por caveolas. Este cambio en el mecanismo de entrada, de clatrinas a caveolas, daría lugar a una reducción de la degradación lisosomal, favoreciendo que los vectores se localicen alrededor del núcleo, lo cual facilita su internalización cuando las células se dividen.

En cualquier caso, aunque el péptido SAP mejora la transfección en las células ARPE-19 (de un 3% a un 6% aproximadamente), es necesaria una mayor eficacia para tratar las enfermedades de la retina. Como ya hemos comentado anteriormente, la incorporación en las formulaciones de una secuencia NLS que favorezca la entrada al núcleo podría ser una interesante estrategia para mejorar la transfección en las células de retina. Subramanian y col.[48] obtuvieron un aumento del 5% al 80% de transfección en células que no se dividen mediante la incorporación de NLS a sus vectores. Sin

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48 Subramanian A, Ranganathan P, Diamond SL. Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. Nat Biotechnol. 1999; 17: 873–877.

embargo, esta estrategia no siempre ha dado lugar a mejoras en la transfección[49], por lo que es necesaria su optimización en cada línea celular y formulación a estudiar.

ESTABILIDAD A CORTO Y LARGO PLAZO DE LAS FORMULACIONES LIOFILIZADAS

Aunque uno de los objetivos de la terapia génica es el desarrollo de vectores eficaces y seguros, no hay que olvidar un aspecto no menos importante, que es la estabilidad. Es preciso que las formulaciones sean física y químicamente estables para conseguir lotes grandes, fáciles de transportar y almacenar. Una importante limitación de las suspensiones acuosas de vectores no virales es su baja estabilidad[50,51,52,53], y las SLNs no son una excepción, observándose en muchos casos un aumento del tamaño de partícula en periodos cortos de tiempo[54]. De hecho, las suspensiones de SLNs utilizadas en este estudio fueron estables sólo durante 1 semana a temperatura ambiente y 1 mes a 4ºC.

Uno de los métodos más utilizados para obtener formulaciones en forma de polvo a partir de formulaciones acuosas es la liofilización. Sin embargo, este proceso somete a

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49 Boulanger C, Di Giorgio C, Vierling P. Synthesis of acridine-nuclear localization signal (NLS) conjugates and evaluation of their impact on lipoplex and polyplex-based transfection. Eur J Med Chem. 2005; 40: 1295-1306.

50 Gustafsson J, Arvidson G, Karlsson G, Almgren M. Complexes between cationic liposomes and DNA visualized by Cryo-Tem. Biochim Biophys Acta. 1995; 1235: 305-312.

51 Tang MX, Szoka FC. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Ther. 1997; 4: 823-832.

52 Mahato RI. Non-viral peptide-based approaches to gene delivery. J Drug Targ. 1999; 7: 249-268.

53 Anchordoquy TJ, Koe GS. Physical stability of nonviral plasmid-based therapeutics. J Pharm Sci. 2000; 89: 289-296.

54 Freitas C, Müller RH. Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN(TM)) dispersions. Int J Pharm. 1998; 168: 221-229.

las formulaciones a dos importantes cambios que pueden resultar en problemas adicionales de estabilidad. En primer lugar, las dispersiones acuosas se congelan, antes de la evaporación del agua a vacío, y a continuación, antes de la administración, debe llevarse a cabo la re-dispersión. El proceso de liofilización de las SLNs, con o sin ADN, en ausencia de un agente estabilizante dio lugar a la agregación de las partículas formando un producto gomoso. La congelación y la re-dispersión pueden ser responsables de la agregación debido a una reducción de la cantidad de agua en la que se encuentran suspendidas las partículas, favoreciendo su concentración[13,55,56]. En este trabajo[57] se ha estudiado el efecto de la liofilización en las características morfológicas y en la capacidad de transfección de los vectores basados en SLNs, así como la estabilidad del producto de la liofilización a corto y largo plazo. Para ello, se han liofilizado por un lado SLNs, que tras ser resuspendidas se han unido al ADN, y por otro, se ha llevado a cabo la liofilización de vectores SLN-ADN. Esta segunda estrategia podría ser más conveniente desde un punto de vista práctico ya que sólo requiere la reconstitución de los vectores antes de su uso en clínica. Puesto que la liofilización puede dañar el ADN[58] y dar lugar a una reducción de la actividad, es necesario llevar a cabo una evaluación exhaustiva de los productos resultantes. Para mejorar la

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55 Allison SD, Molina M, Anchordoquy TJ. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hipotesis. Biochim Biophys Acta. 2000; 1468: 127-138.

56 Molina MDC, Allison SD, Anchordoquy TJ. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: Insight from other structural indicators. J Pharm Sci. 2001; 90: 1445-1455.

57 del Pozo-Rodríguez A, Solinís MA, Gascón AR, Pedraz JL. Short- and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy. Eur J Pharm Biopharm. Accepted, doi: 10.1016/j.ejpb.2008.09.015.

58 Poxon SW, Hughes JA. The effect of lyophilization on plasmid DNA activity. Pharm Dev Technol. 2000; 5: 115-122.

estabilidad, es frecuente utilizar carbohidratos que actúan como lio-protectores y evitan la agregación de las partículas y la desnaturalización de las macromoléculas.

Varios autores han demostrado que los disacáridos son buenos estabilizantes para los vectores lipídicos[55,59,60], y uno de ellos, la trehalosa, se usa frecuentemente como estabilizante de las SLNs liofilizadas[61,62,63,64]. Estos azúcares deben tener una temperatura de transición vítrea alta (T_g), de manera que durante la liofilización la temperatura se mantenga por debajo de ella; de no ser así, la fracción congelada pasaría a estado líquido o gomoso durante el proceso[65]. Además, las muestras liofilizadas en presencia de azúcares con T_g alta, como la trehalosa, muestran menos tendencia a agregarse durante el almacenamiento en comparación con aquellas liofilizadas con azúcares de menor T_g, como la sacarosa o la glucosa[66]. De hecho, cuando nosotros utilizamos el monosacárido glucosa como estabilizante, los liofilizados mostraron aspecto gomoso; únicamente las SLNs liofilizadas con glucosa al 5% presentaron aspecto de

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59 Anchordoquy TJ, Carpenter JF, Kroll DJ. Maintenance of transfection rates and physical characterization of lipid/DNA complexes after freeze-drying and rehydration. Arch Biochem Biophys. 1997; 348: 199-206.

60 Allison SD, Anchordoquy TJ. Mechanisms of protection of cationic lipid-DNA complexes during lyophilization. J Pharm Sci. 2000; 89: 682-691.

61 Schwarz C, Mehnert W. Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles (SLN). Int J Pharm. 1997; 157: 171-179.

62 Zimmermann E, Muller RH, Mader K. Influence of different parameters on reconstitution of lyophilized SLN. Int J Pharm. 2000; 196: 211-213.

63 Cavalli R, Caputo O, Carlotti ME, Trotta M, Scarnecchia C, Gasco MR. Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. Int J Pharm. 1997; 148: 47-54.

64 Heiati H, Tawashi R, Phillips NC. Drug retention and stability of solid lipid nanoparticles containing azidothymidine palmitate after autoclaving, storage and lyophilization. J Microencapsul. 1998; 15: 173-184.

65 Hinrichs WLJ, Prinsen MG, Frijlink HW. Inulin glasses for the stabilization of therapeutic proteins. Int J Pharm. 2001; 215: 163-174.

66 Molina MD, Armstrong TK, Zhang Y, Patel MM, Lentz YK, Anchordoquy TJ. The stability of lyophilized lipid/DNA complexes during prolonged storage. J Pharm Sci. 2004; 93: 2259-2273.

polvo, pero después de 2 días a temperatura ambiente se volvieron también gomosas. Sin embargo, la liofilización en presencia de trehalosa dio lugar a muestras con aspecto fibroso o de polvo, por lo que se seleccionó este disacárido como estabilizante de las SLNs.

Tanto las SLNs como los sistemas SLN-ADN liofilizados con soluciones de trehalosa al 5 o al 10% mostraron aspecto de polvo y fueron fácilmente redispersados mediante agitación en vórtex, aunque en las formulaciones estabilizadas con trehalosa al 5% aparecieron micropartículas. Además, la liofilización dio lugar a un descenso de la carga superficial y a un aumento del tamaño de partícula. El descenso en la carga superficial podría deberse a dos fenómenos: la base de Schiff[67] o la hipótesis del reemplazamiento del agua[68,69]. El fenómeno de la base de Schiff hace referencia a la reacción entre los grupos reductores de los azúcares y los grupos aminos del DOTAP. Esto ocurre cuando se usan azúcares reductores, o en el caso de que un azúcar no reductor sea hidrolizado dando lugar a la aparición de otros reductores. Sin embargo, la trehalosa es un disacárido no reductor muy resistente a la hidrólisis, por lo que la base de Schiff parece no ser responsable de la reducción detectada en la carga superficial. Por lo tanto, en nuestro caso la reducción de la carga superficial se deberá más bien a la hipótesis del reemplazamiento del agua; es decir, a la formación de puentes de hidrógeno entre la trehalosa y los vectores SLN-ADN. Esos puentes pueden tener lugar

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67 Hinrichs WLJ, Sanders NN, De Smedt SC, Demeester J, Frijlink HW. Inulin is a promising cryo- and lyoprotectant for PEGylated lipoplexes. J Control Release. 2005; 103: 465-479.

68 Crowe JH, Crowe LM. Preserving dry biomaterials: the water replacement hypothesis. Part 1. BioPharm. 1993; 6: 28-37.

69 Crowe JH, Crowe LM. Preserving dry biomaterials: the water replacement hypothesis. Part 2. BioPharm. 1993; 6: 40-43.

con los grupos de cabeza de los lípidos catiónicos o con el ADN. Los grupos de cabeza de los lípidos catiónicos presentan grupos amonio que son responsables de la carga positiva. Cuando los azúcares forman enlaces con esos grupos la carga superficial disminuye y la unión del ADN a través de interacciones electrostáticas es más débil, resultando en una menor condensación del plásmido. Esta menor condensación es responsable del aumento del tamaño y la disminución del potencial zeta, puesto que las cargas superficiales negativas del ADN están más expuestas.

La disminución de la carga superficial de las SLNs puede dar lugar una menor capacidad de unión del ADN. Sin embargo, tras la liofilización, las nanopartículas mantuvieron esa capacidad de unión, necesaria para la transfección. Por otro lado, la liofilización de los vectores SLN-ADN indujo cambios en la topología del plásmido, de manera que el ADN fue parcialmente dañado durante el proceso de liofilización, disminuyendo la presencia de la isoforma con mayor actividad biológica, la isoforma de ADN superenrollado (SC), aunque sin llegar a desaparecer completamente.

Una vez caracterizadas las muestras liofilizadas, es necesario conocer su capacidad de transfección. Varios trabajos han relacionado el mantenimiento del tamaño de los sistemas lipídicos y la eficacia de transfección[56,59,60,66,70,71] pero en nuestro trabajo, la liofilización no indujo cambios en los niveles de transfección a pesar del aumento del tamaño. Sin embargo, es importante tener en cuenta que en los estudios citados los sistemas lipídicos evaluados fueron liposomas y no SLNs. En cualquier caso, no

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70 Li B, Li S, Tan YD, Stolz DB, Watkins SC, Block LH, et al. Lyophilization of cationic lipid-protamine-DNA (LPD) complexes. J Pharm Sci. 2000; 89: 355-364.

71 Seville PC, Kellaway IW, Birchall JC. Preparation of dry powder dispersions for non-viral gene delivery by freeze-drying and spray-drying. J Gene Med. 2002; 4: 428-437.

conviene olvidar que el cambio en el tamaño de partícula apreciado tras la liofilización es un factor importante a tener en cuenta a la hora de administrar estos vectores "*in vivo*", ya que puede condicionar la vía de administración, la biodistribución y la eficacia. Los cambios en la carga superficial tampoco se tradujeron en diferencias en la eficacia de transfección "*in vitro*".

Una vez demostrado que las formulaciones liofilizadas mantienen la capacidad de transfección, se estudió la estabilidad a corto y largo plazo de las formulaciones liofilizadas con trehalosa al 10%. Para ello, las SLNs se envasaron en viales de borosilicato ámbar y los vectores SLN-DNA en crioviales de poli-propileno, para evitar la unión del ADN al vidrio[72,73]. Las muestras se almacenaron en cámaras climáticas para controlar las condiciones de temperatura y humedad objeto de estudio: 25ºC/60%HR (estudio de estabilidad a largo plazo), 30ºC/65%HR (estudio de estabilidad a medio plazo) y 40ºC/75%HR (estudio de estabilidad acelerado o a corto plazo).

El comportamiento de las SLNs liofilizadas y de los vectores SLN-ADN liofilizados durante el periodo de almacenamiento fue diferente. Las SLNs liofilizadas se mantuvieron estables físicamente durante 6 meses a 30ºC/65%HR y durante 9 meses a 25ºC/60%HR; sin embargo, a 40ºC/75%HR la estabilidad disminuyó desde el primer mes. La transfección se mantuvo e incluso aumentó con las SLNs almacenadas a 25ºC/60%HR y 30ºC/65%HR, pero no fue así en el caso del almacenamiento a 40ºC/75%HR. Los cambios en el tamaño no parecen ser responsables de estas

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72 Nanassy OZ, Haydock PV, Reed MW. Capture of genomic DNA on glass microscope slides. Anal Biochem. 2007; 365: 240–245. 661

73 Vogelstein B, Gillespie D. Preparative and analytical purification of DNA from agarose. Proc Natl Acad Sci USA. 1979; 76: 615–619.

diferencias en la transfección, ya que, como se aprecia en la Figura 18, cuando las formulaciones se almacenaron a 25ºC/60%HR el aumento en la transfección se observó a los 6 y 9 meses, y a esos tiempos no se detectaron cambios en el tamaño de partícula. Asimismo, cuando las muestras se almacenaron a 30ºC/65%HR el incremento en los niveles de transfección se observó a los 3, 6 y 9 meses, y los cambios en el tamaño sólo ocurrieron a los 3 y 9 meses de almacenamiento.

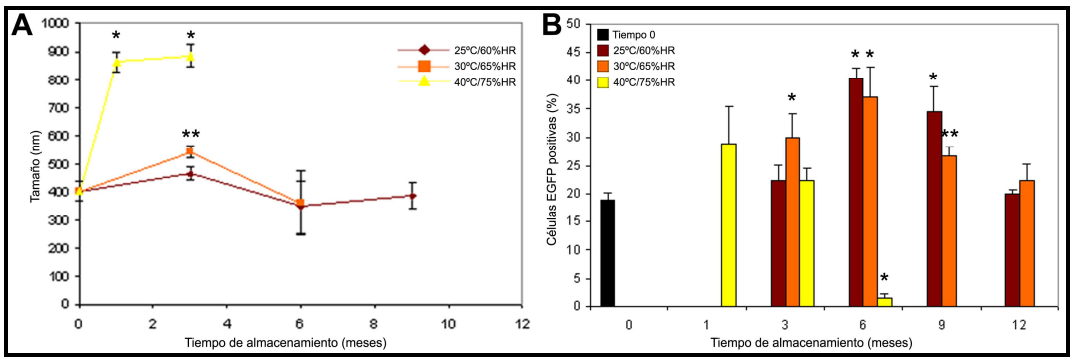

Figura 9. Tamaño (A) y niveles de transfección (B) obtenidos a diferentes tiempos del almacenamiento de las SLNs liofilizadas en presencia de trehalosa al 10%. (Barras de error = D.E., n=3) *: $p < 0.01$ frente a tiempo 0; **: $p < 0.05$ frente a tiempo 0.

Las diferencias en los niveles de transfección parecen relacionadas con la condensación del ADN sobre las nanopartículas. Cuando las SLNs se almacenaron a 25ºC/60%HR y 30ºC/65%HR, mostraron una capacidad de condensación algo menor que la que se obtiene con las SLNs no liofilizadas, lo cual favorece la liberación del plásmido y la transfección "*in vitro*". Sin embargo, en el caso de las SLNs almacenadas a 40ºC/75%HR, la condensación fue mucho menor, siendo probablemente insuficiente para transportar el ADN a través del citoplasma y dirigirlo al núcleo, lo cual dificulta la posterior entrada y disminuye la transfección.

Los vectores SLN-ADN liofilizados sufrieron una importante pérdida de la capacidad de transfección en todas las condiciones de almacenamiento utilizadas. La reducción de la transfección parece estar relacionada con la fuerte unión del ADN a las SLNs, que dificulta su liberación.

El proceso de liofilización no redujo la viabilidad celular.

Los resultados muestran que es posible obtener SLNs liofilizadas estables durante 6 y 9 meses cuando se almacenan a 30ºC/65%HR y 25ºC/60%HR, respectivamente. Sin embargo, son inestables en condiciones más extremas (40ºC/75%HR). Además, la liofilización de los vectores SLN-ADN conduce a una pérdida de la capacidad de transfección.

TRANSFECCIÓN "*IN VIVO*"

Una vez caracterizados los vectores SLN-ADN, y estudiado su comportamiento intracelular, además de conocer su estabilidad a corto y largo plazo, se estudió su capacidad de transfección "*in vivo*". Para ello, se administraron los vectores a ratones Balb/c por vía intravenosa a través de la vena de la cola y se analizaron el hígado, bazo y pulmones para demostrar la presencia de EGFP mediante inmunofluorescencia.

La captación por los órganos y tejidos del organismo va a estar condicionada por el tamaño de los mismos. Si se administran por vía intravenosa, el tamaño va a afectar a la salida de la circulación, ya que según el órgano o tejido y su estado, la estructura de la pared capilar varía. Por ejemplo, la capa de células endoteliales en tejidos inflamados y tumores presenta poros de entre 100 nm y 700 nm, según la localización, lo que permite que las partículas menores de esos tamaños salgan del torrente circulatorio y se

distribuyan en esos tejidos[74]. Mahato y col.[75] y Bragonzi y col.[76] han demostrado que los liposomas de 400 nm o mayores producen niveles de transfección en el pulmón más altos que los liposomas de 100 nm o menores. Esto se atribuye a la captación por los capilares pulmonares de las partículas de mayor tamaño. Además, cuando la administración tiene lugar por vía oral, el tamaño de partícula condiciona la captación por las placas de Peyer[77].

La carga superficial o potencial zeta de los vectores no virales también es determinante en el proceso de transfección. La carga superficial positiva es ventajosa en terapia génica, ya que facilita la interacción con la superficie celular cargada negativamente. "*In vivo*", la carga superficial condiciona además el tiempo de permanencia en la circulación, la distribución tisular y la captación celular. Sakurai y col.[78] y Eliyahu y col.[79] han demostrado que los lípidos catiónicos interaccionan con los eritrocitos dando lugar a la formación de agregados que se acumulan en el pulmón, debido a que ese es el primer entramado capilar que se encuentran en el proceso de

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74 Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, et al. Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. Cancer Res. 1995; 55: 3752-3756.

75 Mahato RI, Anwer K, Tagliaferri F, Meaney G, Leonard P, Wadhwa MS, et al. Biodistribution and gene expression of lipid/plasmid complexes after systemic administration. Hum Gene Ther. 1998; 9: 2083-2099.

76 Bragonzi A, Dina G, Villa G, Calori G, Biffi A, Bordignon C, et al. Biodistribution and transgene expresion with nonviral cationic vector/DNA complexes in the lungs. Gene Ther. 2000; 7: 1753-1760.

77 Delie F. Evaluation of nano- and microparticle uptake by the gastrointestinal tract. Adv Drug Deliv Rev. 1998; 34: 221-233.

78 Sakurai F, Nishioka T, Saito H, Baba T, Okuda A, Matsumoto O, et al. Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. Gene Ther. 2001; 8: 677-686.

79 Eliyahu H, Joseph A, Schillemans JP, Azzam T, Domb AJ, Barenholz Y. Characterization and in vivo performance of dextran-spermine polyplexes and DOTAP/cholesterol lipoplexes administered locally and systematically. Biomaterials. 2007; 28: 2339-2349.

distribución por el organismo. Este hecho no es sólo una limitación en cuanto a la llegada de los vectores a otros órganos diana, sino que también puede dar lugar a serios problemas si se produce, por ejemplo, una embolia pulmonar. Además, ha sido documentado que las partículas exógenas son opsonizadas por proteínas plasmáticas como un proceso natural mediado por el sistema inmune[80], lo que lleva a su captación por células del sistema reticuloendotelial (SRE) y un rápido aclaramiento del torrente sanguíneo. Las nanopartículas también pueden activar el sistema del complemento, dando lugar a su eliminación a través del sistema fagocítico mononuclear. Aunque las partículas más grandes parecen tener mayor capacidad de activación del sistema del complemento, la carga superficial positiva también parece tener correlación con esa activación[81].

Con el fin de reducir las interacciones con los componentes sanguíneos se vienen utilizando distintas estrategias que incluyen: el desarrollo de nuevos lípidos catiónicos resistentes al suero[82], la inclusión de lípidos neutros en las formulaciones[78], la estabilización de los vectores con agentes como las poliaminas[83] o el sulfato de

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80 Owens DE 3rd, Peppas NA. Opsonization, biodistribution and pharmacokinetics of polymeric nanoparticles.. Int J Pharm. 2006; 307: 93-102.

81 Moghimi SM, Szebeni J. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog Lipid Res. 2003; 42: 463-478.

82 Lewis JG, Lin KY, Kothavale A, Flanagan WM, Matteucci MD, DePrince RB, et al. A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. Proc Natl Acad Sci USA. 1996; 93: 3176-3181.

83 Escriou V, Ciolina C, Lacroix F, Byk G, Scherman D, Wils P. Cationic lipid-mediated gene transfer: effect of serum on cellular uptake and intracellular fate of lipopolyamine/DNA complexes. Biocim Biophys Acta. 1998; 1368: 276-288.

protamina[84] y la creación de una barrera sobre la carga positiva mediante la estabilización estérica que proporcionan las cadenas de polietilenglicol (PEG)[81,85,86]. Con esta última estrategia las partículas obtenidas se conocen como partículas pegiladas. La adición de cadenas de PEG se puede llevar a cabo mediante adsorción física, incorporación durante la producción de los vectores o unión covalente a alguno de los componentes de la formulación. En este trabajo se ha recurrido a la segunda opción, incorporando las cadenas de PEG mediante la inclusión de Tween-80 en la composición de las nanopartículas. Esas cadenas, por un lado evitan la unión de cada molécula de ADN a más de una partícula y la formación de agregados[87], y por otro lado, neutralizan el exceso de cargas positivas reduciendo la interacción con los componentes sanguíneos. Sin embargo, la estrategia de la estabilización estérica tiene un importante inconveniente: la interacción con las células a transfectar se ve reducida como consecuencia de la neutralización de la carga positiva.

La administración intravenosa de las SLNs dio lugar a transfección en tejido hepático y esplénico hasta 7 días después de la administración (Figura 19). Sin embargo, no se detectó EGFP a nivel pulmonar, a pesar de que en otros estudios tras la administración intravenosa de sistemas catiónicos, aproximadamente el 80% de la dosis se acumula en

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84 Sorgi FL, Bhattacharya S, Huang. Protamine sulfate enhances lipid-mediated gene transfer. Gene Ther. 1997; 4: 961-968.

85 Tranchant I, Thompson B, Nicolazzi C, Mignet N, Scherman D. Physicochemical optimisation of plasmid delivery by cationic lipids. J Gene Med. 2004; 6: S24-S35.

86 Harvie P, Frances MW, Marcel BB. Use of poly(ethyleneglycol)-lipid conjugates to regulate the surface attributes and transfection activity of lipid-DNA particles. J Pharm Sci. 89; 2006: 652-663.

87 Liu F, Yang JP, Huang L, Liu DX. Effect of non-ionic surfactants on the formation of DNA/emulsion complexes and emulsion-mediated gene transfer. Pharm Res. 1996; 13: 1642-1646.

los pulmones dando lugar a la consecuente expresión génica en ese tejido[88,89,78]. Como ya hemos comentado anteriormente, la acumulación en los pulmones se relaciona con la formación de agregados cuando los vectores interaccionan con componentes sanguíneos. En este trabajo, los vectores mantienen su carácter catiónico, ya que las cadenas de PEG del Tween-80 no son capaces de neutralizar totalmente las cargas positivas. Aún así, la barrera estérica proporcionada parece suficiente para evitar en parte la interacción con componentes sanguíneos, lo cual se ve confirmado por el hecho de obtener transfección "*in vitro*" en presencia de suero.

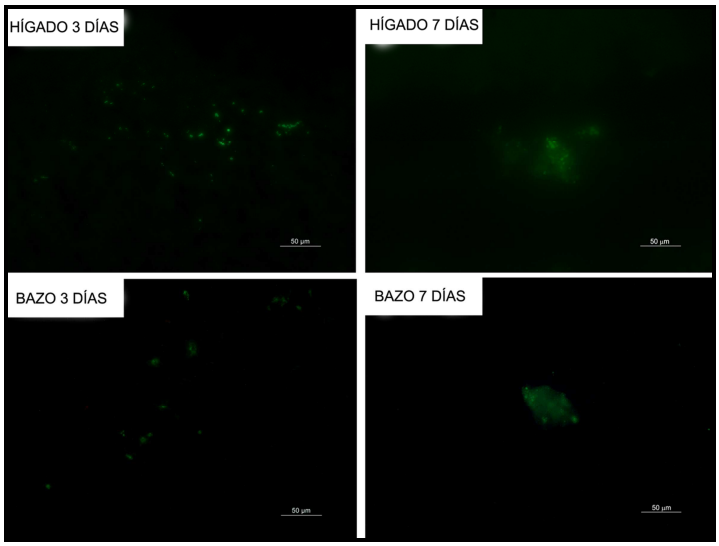

Figura 10. Imágenes de dos secciones de hígado y bazo de ratón después del marcaje de la proteína EGFP mediante inmunofluorescencia. Las secciones fueron extraídas de los ratones sacrificados a los 3 y 7 días tras la administración intravenosa de los vectores SLN-ADN (dosis: 60 μg ADN).

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88 Kichler A. Gene transfer with modified polyethylenimines. J Gene Med. 2004; 6: S3-S10.

89 Eliyahu H, Servel N, Domb AJ, Barenholz Y. Lipoplex-induced hemagglutination: potential involvement in intravenous gene delivery. Gene Ther. 2002; 9: 850-858.

Aunque se considera que las partículas pegiladas son ignoradas por las opsoninas, varios autores han demostrado el aclaramiento de partículas modificadas con PEG por parte de los macrófagos del hígado o el bazo[90,91,92,93,94,95] (SRE), lo cual podría explicar la expresión de EGFP observada en estos órganos tras la administración sistémica de los vectores SLN-ADN.

El aclaramiento a través del SRE puede ser útil cuando el hígado o el bazo son el órgano diana, por ejemplo, en la caso de las vacunas de ADN o para el tratamiento de tumores en esos órganos. El bazo se ha estudiado más frecuentemente como órgano diana para las vacunas basadas en ADN[96,97], pero la llegada de genes al hígado puede

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90 Allen TM, Hansen C, Martin F, Redemann C, Yauyoung A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives Invivo. Biochim Biophys Acta. 1991; 1066: 29-36.

91 Laverman P, Brouwers AH, Dams ETM, Oyen WJG, Storm G, Van Rooijen N, et al. Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose. J Pharmacol Exp Ther. 2000; 293: 996-1001.

92 Laverman P, Boerman OC, Oyen WJG, Corstens FHM, Storm G. In vivo applications of PEG liposomes: Unexpected observations. Crit Rev Ther Drug. 2001; 18: 551-566.

93 Laverman P, Carstens MG, Boerman OC, Dams ETM, Oyen WJG, Van Rooijen N, et al. Factors affecting the accelerated blood clearance of polyethylene glycol-liposomes upon repeated injection. J Pharmacol Exp Ther. 2001; 298: 607-612.

94 Utkhede DR, Tilcock CP. Studies upon the toxicity of polyethylene glycol coated lipid vesicles: Acute hemodynamic effects, pyrogenicity and complement activation. J Liposome Res. 1998; 8: 537-550.

95 Woodle MC, Matthay KK, Newman MS, Hidayat JE, Collins LR, Redemann C, et al. Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. Biochim Biophys Acta. 1992; 1105: 193-200.

96 Nakamura J, Fumoto S, Kawanami R, Kodama Y, Nishi J, Nakashima M, et al. Spleen-selective gene transfer following the administration of naked plasmid DNA onto the spleen surface in mice. Biol Pharm Bull. 2007; 30: 941-945.

97 Tupin E, Poirier B, Bureau MF, Khallou-Laschet J, Vranckx R, Caligiuri G, et al. Non-viral gene transfer of murine spleen cells achieved by in vivo electroporation. Gene Ther. 2003; 10: 569-579.

ser útil no sólo en el caso de vacunas[98], sino también para el tratamiento de enfermedades como el cáncer[99] o la hepatitis B[100] y para la creación de un órgano de depósito a partir del cual se secreten al torrente sanguíneo proteínas ausentes en el organismo[101].

En conclusión, podemos decir que las SLNs son vectores no virales capaces de transfectar tanto "*in vitro*" como "*in vivo*", y cuya estabilidad cuando son liofilizadas permite mantenerlas durante 6 meses a 30ºC/65%HR o 9 meses a 25ºC/60%HR. Aunque nuestras formulaciones han mostrado capacidad de transfección cuando son administradas por vía intravenosa en ratones, es necesario el desarrollo de sistemas más eficaces, que permitan obtener niveles de transfección mayores y durante más tiempo. Además, son necesarios nuevos estudios para saber si otros órganos pueden ser también transfectados y para evaluar otras vías de administración. Para ello, se pueden considerar varias estrategias. Por un lado, la incorporación de ligandos en las formulaciones puede resultar útil para dirigir los vectores no virales a diferentes órganos en función de la enfermedad que se quiera tratar; por ejemplo, secuencias RGD para

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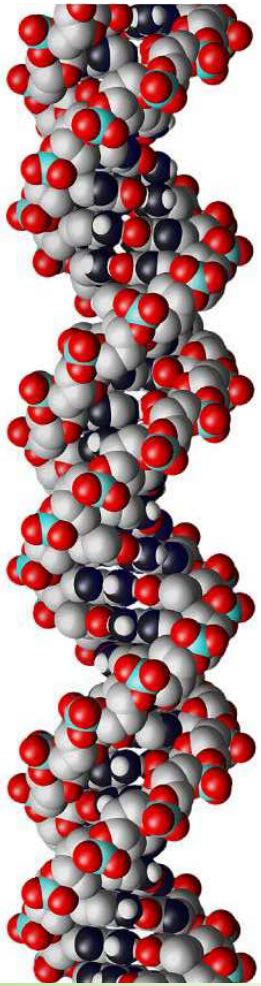
98 Raska M, Moldoveanu Z, Novak J, Hel Z, Novak L, Bozja J, et al. Delivery of DNA HIV-1 vaccine to the liver induces high and long-lasting humoral immune responses. Vaccine 2008; 26: 1541-1551.

99 Tang H, Tang XY, Liu M, Li X. Targeting alpha-fetoprotein represses the proliferation of hepatoma cells via regulation of the cell cycle. Clin Chim Acta. 2008; 394: 81-88.

100 Zhang Y, Rong Qi X, Gao Y, Wei L, Maitani Y, Nagai Y. Mechanisms of co-modified liver-targeting liposomes as gene delivery carriers based on cellular uptake and antigens inhibition effect. J Control Release. 2007; 117: 281-290.

101 Mango RL, Xu L, Sands MS, Vogler C, Seiler G, Schwarz T, et al. Neonatal retroviral vector-mediated hepatic gene therapy reduces bone, joint, and cartilage disease in mucopolysaccharidosis VII mice and dogs. Mol Genet Metab. 2004; 82: 4-19.

neuronas o células endoteliales de tumor, transferrina o ácido fólico para células tumorales, lactoferrina para el cerebro, o azúcares para los hepatocitos. Por otro lado, la inclusión en los vectores de moléculas que han demostrado mejorar la capacidad de transfección "*in vitro*", como las señales de localización nuclear (NLS), puede dar lugar a importantes beneficios en el campo de la terapia génica.



CONCLUSIONES

Se han desarrollado nanopartículas sólidas lipídicas (SLNs) como sistemas no virales de administración de ADN con capacidad de transfección tanto "*in vitro*" como "*in vivo*". Los resultados obtenidos en esta memoria experimental han permitido obtener las siguientes conclusiones:

6. La eficacia de transfección "*in vitro*" de las SLNs depende de la proporción del lípido catiónico DOTAP y de la relación DOTAP:ADN utilizada para elaborar los complejos SLN-ADN. Las formulaciones compuestas por Precirol® ATO 5, DOTAP al 0.4% y Tween-80 al 0.1% son las que proporcionan mayor nivel de transfección. Para obtener una eficiencia de unión del ADN a las SLNs del 100% es necesaria una relación DOTAP:ADN de al menos 4:1. A medida que la relación DOTAP:ADN disminuye, la condensación del ADN también disminuye, produciéndose un aumento del tamaño, una reducción de la carga superficial y una disminución de la protección del ADN frente a la enzima DNasa I. La relación DOTAP:ADN óptima es 5:1.

7. La capacidad de transfección "*in vitro*" de los vectores depende de la línea celular, siendo menor en las células ARPE-19 que en las HEK293. Las diferencias se explican en función del mecanismo de internalización y de la distribución intracelular. La internalización de los vectores en las células HEK293 tiene lugar principalmente por un proceso de endocitosis mediada por caveolas, mientras que en las células ARPE-19 ocurre a través de endocitosis mediada por clatrinas. Además, la entrada más lenta de los vectores en las células de retina y la menor velocidad de división de estas células justifica el retraso observado en la expresión de la proteína verde fluorescente en la línea celular ARPE-19.

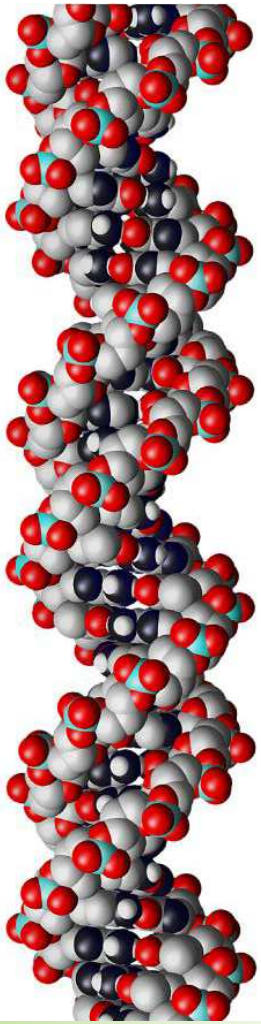
8. La incorporación del péptido de penetración celular SAP en los vectores produce un incremento en el nivel de transfección en las dos líneas celulares estudiadas, HEK293 y ARPE-19. En las células HEK293 el incremento en la transfección se justifica por un incremento en la entrada de los vectores en el interior de las células. En las células ARPE-19, el incremento en la capacidad de transfección se debe a que el péptido SAP favorece la endocitosis mediada por caveolas. Este cambio en el mecanismo de entrada, de clatrinas a caveolas, daría lugar a una reducción de la degradación lisosomal, favoreciendo que los vectores se localicen alrededor del núcleo, lo cual facilita su internalización cuando las células se dividen.

9. La liofilización tanto de las SLNs como de los complejos SLN-ADN en presencia de soluciones de trehalosa al 5 ó al 10% da lugar a formulaciones con aspecto de polvo fácilmente re-dispersables mediante agitación, que mantienen la capacidad de transfección.

10. Las SLNs liofilizadas en presencia de trehalosa al 10% se mantienen estables físicamente durante 6 meses a 30ºC/65%HR y durante 9 meses a 25ºC/60%HR; sin embargo, a 40ºC/75%HR la estabilidad disminuye desde el primer mes. La transfección se mantiene e incluso aumenta con las SLNs almacenadas a 25ºC/60%HR y 30ºC/65%HR, pero disminuye cuando se almacenan a 40ºC/75%HR. Las diferencias en los niveles de transfección se asocian a un cambio en el grado de condensación del ADN durante el almacenamiento.

11. La liofilización de los vectores SLN-ADN conduce a una pérdida de la capacidad de transfección. Esta reducción del nivel de transfección parece estar relacionada con un incremento en la unión del ADN a las SLNs, que dificulta su liberación.

12. Los vectores no virales basados en SLNs son capaces de transfectar “in vivo” tras la administración intravenosa a ratones Balb/c, dando lugar a la expresión de proteína en el hígado y en el bazo, que se mantiene durante al menos 7 días. La administración de estos vectores SLN-ADN por vía intravenosa no induce la expresión de proteína en los pulmones.



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