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ORIGINAL ARTICLE

Male Infertility

Local renin angiotensin system and sperm DNA fragmentation

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The renin angiotensin system (RAS) appears to influence male fertility at multiple levels. In this work, we analyzed the relationship between the RAS and DNA integrity. Fifty male volunteers were divided into two groups (25 each): control (DNA fragmentation ≤20%) and pathological (DNA fragmentation >20%) cases. Activities of five peptidases controlling RAS were measured fluorometrically: prolyl endopeptidase (which converts angiotensin [A] I and A II to A 1–7), neutral endopeptidase (NEP/CD10: A I to A 1–7), aminopeptidase N (APN/CD13: A III to A IV), aminopeptidase A (A II to A III) and aminopeptidase B (A III to A IV). Angiotensin-converting enzyme (A I to A II), APN/CD13 and NEP/CD10 were also assessed by semiquantitative cytometry and quantitative flow cytometry assays, as were the receptors of all RAS components: A II receptor type 1 (AT1R), A II receptor type 2 (AT2R), A IV receptor (AT4R or insulin-regulated aminopeptidase [IRAP]), (pro)renin receptor (PRR) and A 1–7 receptor or Mas receptor (MasR) None of the enzymes that regulate levels of RAS components, except for APN/CD13 (decrease in fragmented cells), showed significant differences between both groups. Micrographs of RAS receptors revealed no significant differences in immunolabeling patterns between normozoospermic and fragmented cells. Labeling of AT1R (94.3% normozoospermic *vs* 84.1% fragmented), AT4R (96.2% *vs* 95.3%) and MasR (97.4% *vs* 87.2%) was similar between the groups. AT2R (87.4% normozoospermic *vs* 63.1% fragmented) and PRR (96.4% *vs* 48.2%) were higher in non-fragmented spermatozoa. These findings suggest that fragmented DNA spermatozoa have a lower capacity to respond to bioactive RAS peptides.

INTRODUCTION

In Western societies, approximately one of six couples of reproductive age is infertile, with male factor infertility accounting for 50% of all cases. To resolve such male fertility problems, a large number of couples seek medical help, and intracytoplasmic sperm injection (ICSI) is the primary approach used to achieve pregnancy. The study of male infertility factors has usually been based on the analysis of semen, using the World Health Organization (WHO) guidelines.[1] Although not included in this manual, DNA fragmentation has become an important predictor of sperm quality in the last decade. Indeed, DNA fragmentation has become an important marker of sperm quality because of its possible relationship with various critical parameters, such as live and assisted reproduction, embryo-zygote development and quality, implantation, abortion and newborn health.[2–8] Although male fertility status and semen quality can be assessed through seminogram analysis, this method does not detect the presence of DNA fragmentation in spermatozoa. It is estimated that, for 10%–15% of sterile men, these alterations are present in the genetic material of their gametes, whereas parameters of concentration, mobility and sperm morphology are within normal limits. During the normal fertilization process, the female reproductive system has selective mechanisms against DNA-damaged spermatozoa. However, this property is not invoked in *in vitro* fertilization (IVF) or ICSI procedures. Moreover, it is important to emphasize that spermatozoa with DNA fragmentation are capable of fertilization. Therefore, it would seem reasonable to suggest that assisted reproduction techniques should compensate for deterioration in sperm chromatin integrity, especially when ICSI is used and only one morphologically normal spermatozoon is selected for microinjection. In addition, screening of morphologically normal spermatozoa for ICSI and the selection of good-quality embryos for transfer in IVF/ICSI will reduce the potentially adverse effects of sperm DNA damage on the outcome of assisted reproduction. Thus, biomarkers to assist in the prediction or assessment of male infertility would be a great enhancement to its treatment and could be used as complementary tests to spermiograms, improving diagnostic or prognostic power. Furthermore, they would allow the selection of the best spermatozoa to be used in assisted reproduction techniques.

To obtain additional information regarding seminal quality, it is interesting to compare whether sperm DNA fragmentation is related to possible alterations in peptide metabolism, both in the spermatozoon and in the seminal fluid, not only to increase knowledge of the molecular mechanisms underlying sperm function and regulation, but also to describe possible biomarkers of sperm integrity and function. In this regard, the relationship

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between various fertility parameters (including gametes and sperm motility) and levels and activities of various peptide systems, such as opioidergic (neutral aminopeptidase: aminopeptidase N [APN/CD13]), basic aminopeptidase, neutral endopeptidase (NEP/CD10), prolyl endopeptidase (PEP), and μ, δ, and κ-opioid receptors[9–12] and oxytocinergic (PEP and cys-aminopeptidase)[13–16] systems, has been described. The findings suggest that these factors may control reproductive function at multiple sites, and exert direct action on spermatozoa. In recent years, research has proven the presence of a local renin angiotensin system (RAS) in gametes, with an important role in the regulation of both male reproduction and female reproduction;[17,18] nevertheless, the extent of its function is not fully understood. The presence and distribution of RAS components in spermatozoa and the activity of angiotensin (A) suggest that this system regulates reproductive function, directly influencing sperm fertility.[19] Our research group has recently shown that embryos with higher implantation potential are derived from sperm samples with a higher percentage of testicular angiotensin-converting enzyme (tACE)-positive cells and fewer enzyme molecules per spermatozoon on their surface membrane. On the basis of these findings, we propose that the tACE may be used to help embryologists select better semen samples to obtain high-quality blastocysts during IVF.[20] In this work, we present the relationship between the percentage of DNA fragmentation and different components of the local sperm RAS. The components analyzed in this study are as follows: PEP (which converts angiotensin I [A I] and angiotensin II [A II] to angiotensin 1–7 [A 1–7]), NEP (A I to A 1–7), APN/CD13 (angiotensin III [A III] to angiotensin IV [A IV]), aminopeptidase A (APA; A II to A III), aminopeptidase B (APB; A III to A IV), and angiotensin-converting enzyme (ACE/CD143; A I to A II), as well as the A II receptor type 1 (AT1R), A II receptor type 2 (AT2R), A 1–7 receptor or Mas receptor (MasR), A IV receptor (AT4R) and (pro)renin receptor (PRR). By comparing levels of complete RAS in normal and fragmented spermatozoa, we could determine if any of the components can be used as a diagnostic biomarker for reproductive success.

PARTICIPANTS AND METHODS

Ethical considerations
This study was approved by the Ethics Committee of the University of the Basque Country (UPV/EHU), Leioa, Spain (CEISH/61/2011) and Clinical Research Ethics Committee (CEIC) of the Basque Health Service/Osakidetza (CEIC E14/42). All semen samples were obtained from male partners of women who had undergone intrauterine insemination (IUI) cycles at the Basque Biobank (Basque Country, Spain). Sperm samples for research were obtained after the patients provided written informed consent.

Patients, semen analysis, and seminal preparation
The samples used for the trial were the leftovers from the IUI process. Patients with insufficient semen volume or sperm concentration for the study and IUI were eliminated because the latter was the priority. Fifty patients with an average age of 37.4 (standard deviation [s.d.]: 4.4, range: 29–46) years were included in this study and divided into two groups (*n*=25 each): normal (DNA fragmentation ≤20%) and pathological (DNA fragmentation >20%) cases. Power analysis was performed (alpha = 0.05; beta = 0.30; power of 70%).

The sperm DNA fragmentation study was performed on raw samples. The samples used for the study of peptidases and RAS

components were frozen. These samples were analyzed on consecutive days but always under identical experimental conditions. As a control group, DNA fragmentation at ≤20% was used because some studies show that 20% is a good indicator of the cut-off value for pathological-degree fragmentation.[21] In the present study, the degree of fragmentation in the control group was low (maximum: 11.5%), whereas it was always higher than 20% (minimum: 21.7%, maximum: 29.6%) in the pathological group. The difference between the two groups was evident.

The semen samples were collected in sterile containers on the day of IUI by masturbation in the hospital after 2 days to 5 days of sexual abstinence. Seminal sample liquefaction was performed at 37°C and in 5% (v/v) CO_2 for 10 min before processing by density gradient centrifugation for insemination.

The semen volume and sperm concentration and motility were measured for each sample, and all the samples were double-examined to determine the sperm concentration and motility in a Makler® Chamber (Sefi Laboratories, Haifa, Israel) by counting at least 200 spermatozoa per replicate. The mean value of homogeneous replicates was used for analysis. Motility was evaluated according to standards of the WHO.[1] In summary, spermatozoa were classified into three different groups: (1) progressive motility (PR), (2) nonprogressive motility (NP), and (3) immotility (IM). Excess spermatozoa remaining after clinical use for IUI procedures were collected for molecular analysis by flow cytometry. The molecular data obtained were related to sperm DNA fragmentation (measured in fresh samples).

Sperm DNA fragmentation: the sperm chromatin dispersion test (SCD) test
The SCD (Halosperm®; Halotech, Madrid, Spain) was used to determine sperm DNA fragmentation. The SCD is an indirect method for quantifying the percentage of spermatozoa with DNA fragmentation and is based on the differential response of sperm nuclei when exposed to slight acid denaturation and subsequent protein lysis. Nuclear fragmentation can be estimated by quantifying the dispersed nuclei of chromatin and condensed nuclei.[22–24]

The overall process requires three critical steps: step a, integration of the sample into inert agarose; step b, acid denaturation of fragmented DNA, and step c, elimination of nuclear proteins by lysis. For step a, the sperm sample was diluted in phosphate buffer solution (PBS) to a maximum of 20×10^6 ml⁻¹; 50 μl was transferred to an Eppendorf tube with 100 μl of melted agarose at 37°C and mixed gently with a micropipette, and the formation of bubbles was prevented. Next, 8 μl of the cell suspension was placed at the center of a sample well ("S"), and covered with a coverslip, and the material was pressed gently to avoid air bubbles and the slide was held horizontally throughout the process. The control was labeled "C". The slides were placed on a cold surface and transferred to 4°C, for 5 min to solidify the agarose, and then the coverslip was removed by gently sliding it off. All subsequent processing was performed at room temperature (22°C). For step b, denaturant agent solution was applied to the well to cover the sample and incubated for 7 min and then removed by tilting without shaking; the samples was dried and placed horizontally; an important step is to remove the reactive material without shaking. For step c, lysis solution was applied to the well, immersing the sample, followed by incubating for 20 min, removing the reagents by tilting as above, washing the slide for 5 min with distilled water, removing the reagents as above, and dehydrating by incubating first with 70% ethanol and then 100% ethanol, for 2 min each.

After these procedures, eosin staining solution (SSA; Halosperm®; Halotech) was applied, incubated for 7 min, and then removed by

tilting. Thiazine staining solution (SSB; Halosperm®; Halotech) was next added to the wells, incubated for 7 min, removed by tilting and dried at room temperature. A minimum of 600 spermatozoa were observed by bright-field microscopy (Scope A1, ZEISS, Oberkochen, Germany) for dispersed chromatin halos that correlate with the percentage of fragmentation. Sperm with fragmented DNA are those with a halo that is smaller than half of the smaller diameter of the nucleus, and those that lack a halo.[25]

Magnetic separation of apoptotic cells by annexin columns

The technique is based on the binding of superparamagnetic Annexin-microbeads to externalized phosphatidylserine (PS) at the outer leaflet of the plasma membrane of sperm with activated apoptosis signaling or membrane damage.[26] If the sample interacts for seconds with a high-powered magnet, the affected sperm will remain attached to the walls of the column while unaffected sperm will flow. This method of separation or molecular filtration is called magnet-activated cell sorting (MACS; Microbead kit of the ANMB, Miltenyi Biotec, Bergisch Gladbach, Germany).

Peptidase activity measurement

Activities of five peptidase were measured by a fluorometric assay: PEP (A I and A II to A 1–7), NEP (A I to A 1–7), APN/CD13 (A III to A IV), APA (A II to A III), and APB (A III to A IV). The assay is based on the fluorescence of products generated from hydrolysis of a specific substrate by each enzyme present in the sample. Regarding APN (Enzyme Commission [E.C.] No. 3.4.11.2), APB (E.C. No. 3.4.11.6) and APA (E.C. No. 3.4.11.21) aminopeptidase activities, different specific aminoacyl-β-naphthylamide derivatives (Sigma Aldrich, St. Louis, MO, USA) were used as substrates. PEP endopeptidase activity was measured with a modified method from Alponti *et al.*[27] with H-Gly-Pro-β-naphthylamide and Z-Gly-Pro-β-naphthylamide (Bachem, Torrance, CA, USA), modified from Zolfaghari *et al.*[28] N-Dansyl-D-Ala-Gly-p-Nitro-Phe-Gly (DAGNPG; Sigma Aldrich,) was used as a fluorogenic substrate for NEP activity measurement, following the method of Florentin *et al.*[29] and modified by Irazusta *et al.*[30] The substrate solutions were prepared in 50 mmol l^{-1} PBS (pH 7.4) containing 0.25 mg ml^{-1} bovine serum albumin (BSA; Sigma Aldrich) for APN (0.5 mmol l^{-1}), APB (0.5 mmol l^{-1}), APA (0.125 mmol l^{-1}), and PEP (0.125 mmol l^{-1}), and in 50 mmol l^{-1} Tris-HCl buffer (pH 7.4) containing 0.25 mg ml^{-1} BSA for NEP. Because of the high similarity between NEP and ACE, 0.005 mmol l^{-1} captopril (Sigma Aldrich) was added to the NEP substrate solution to inhibit ACE. The seminal fraction samples (10–50 µl, depending on each activity) were mixed in triplicate with 1 ml of each substrate solution mixture and incubated for 30 min at 37°C. The enzymatic reaction was stopped by adding 1 ml of 0.1 mol l^{-1} sodium acetate buffer (pH 4.2).

The amount of β-naphthylamine released was determined by measuring the fluorescence intensity in the reaction mixture at 412 nm (with an excitation wavelength of 345 nm) of enzymes assayed with β-naphthylamide derivatives (spectrofluorometer RF540; Shimadzu, Kyoto, Japan). To determine DAGNPG released in the NEP activity assay, the fluorescence intensity was measured at 410 nm and excited at 342 nm. To subtract background fluorescence, 10 mmol l^{-1} Tris HCl (pH 7.4) was used for the blank instead of sample. Relative fluorescence was converted to product in pmol using a standard curve of increasing concentrations of β-naphthylamine. To convert relative fluorescence product in pmol in the NEP activity assay, a standard curve of increasing concentrations of the product and decreasing concentrations of DAGNPG was generated. In all cases, the sample

protein concentration was measured with the Bradford[31] method using BSA as the standard. The measured activities are expressed as units of peptidase activity (UP) per mg protein: UP mg^{-1}, where UP is the amount of enzyme that hydrolyses in 1 pmol of fluorogenic substrate per min.

Determination of levels of APN/CD13, ACE/CD143, NEP/CD10, and receptors by semiquantitative cytometry

To measure levels of APN/CD13, ACE/CD143 and NEP/CD10 as well as those of AT1R, AT2R, AT4R/IRAP, PRR, and MasR in sperm samples, we performed semiquantitative and quantitative flow cytometry assays using the QuantiBRITE™ PE kit (BD Biosciences, San Jose, CA, USA). The same semen samples were simultaneously used for both analyses.

Surplus sperm samples obtained for assaying DNA fragmentation were fixed in suspension with 4% (*w/v*) paraformaldehyde (PFA; Sigma Aldrich), centrifuged (Labofuge 200; Fisher Scientific, Göteborg, Sweden) at 3500*g* for 6 min and washed in PBS. The samples were incubated in blocking buffer (PBS with 10% [*w/v*] fetal bovine serum [FBS]; Biochrom, Cambridge, UK) for 30 min and then with the following primary antibodies: anti-AT1R (extracellular) antibody (rabbit polyclonal antibody to AT1R; AAR011; Alomone Labs, Jerusalem, Israel), anti-AT2R antibody (rabbit polyclonal; ab19134; Abcam, Cambridge, UK), anti-AT4R antibody (Insulin-regulated aminopeptidase [IRAP]; rabbit polyclonal; clone H-133, sc-135229; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-renin receptor (rabbit polyclonal; clone H-85, sc-67390; Santa Cruz Biotechnology), anti-MasR (rabbit polyclonal; AAR-013; Alomone Labs), PE-labeled human ACE monoclonal phycoerythrin (PE mouse anti-human CD143; 344204; BioLegend, San Diego, CA, USA), PE anti-human CD13 (PE mouse anti-human CD13; 555394; BD Biosciences), and PE mouse anti-human CD10 (PE mouse anti-human CD10; 555375; BD Biosciences).

Each of the receptors and enzymes studied was diluted at 1:200 in PBS and incubated overnight at 4°C. The nuclei were stained with 0.5 µg ml^{-1} Hoechst 33258 (Molecular Probes, Eugene, OR, USA). The primary antibody specificity was evaluated with an isotype control antibody (PE mouse IgG1κ isotype control antibody; 400 112; BioLegend) at the same concentration as that of the primary antibody.

To perform quantitative flow cytometry analysis, we plotted a calibration curve using the mean fluorescence intensity (MFI) values obtained from four different populations of PE-conjugated beads with a known number of PE molecules per bead provided by the QuantiBRITE™ PE kit (BD Biosciences). Briefly, the QuantiBRITE™ PE beads were diluted in 500 µl of 1× PBS with azide plus 0.5% (*w/v*) BSA (Sigma Aldrich) and analyzed by flow cytometry. The fluorescence intensity values for the semen samples and different populations of the QuantiBRITE™ PE beads were obtained at the same time using the same settings for fluorescence and compensation.

The fluorescence data from at least 10 000 events were analyzed with a flow cytometer (Gallios™; BD Biosciences). Blue (Hoechst 33258) and red (PE) fluorescence were collected in the FL9 and FL2 sensors, respectively. To ensure that the fluorescence data were from live spermatozoa, we used a discrimination frame around the sperm population on forward (FSC) and side scatter plots (SSC) and then selected Hoechst 33258-positive events. The percentage of PE-positive cells and mean fluorescence of the sperm samples were determined by subtracting the background fluorescence in each histogram from its control. The PE and Hoechst 33258 fluorescence results were analyzed with Summit software (version 4.3; Beckman Coulter Inc., Los Angeles, CA, USA).

Finally, we considered the percentage of receptor- and enzyme-positive cells measured in each semen sample for semi-quantitative flow cytometry analysis. For the quantitative flow cytometry assay, we determined the average number of molecules per spermatozoon, which was extrapolated from a calibration curve obtained from the populations of PE-conjugated beads, because the PE:Ab ratio of our primary antibody was 1:1.

Determination of receptor expression by immunocytochemistry
Protein detection was performed using indirect immunofluorescence. This method requires the consecutive use of two antibodies: a primary antibody that binds to the target protein and a secondary antibody bound to a fluorochrome, which specifically detects the primary antibody.

Spermatozoa previously fixed in 2% paraformaldehyde (*v*/*v*) were washed and resuspended in 1× PBS. They were then spread on cover slips previously treated with poly-L-lysine and dried.

The samples were permeabilized with 1% (*v*/*v*) Triton® x-100 (Sigma Aldrich) in 1× PBS for 10 min under agitation. The Triton® x-100 was removed, and the samples were washed three times for 5 min with 1× PBS. Subsequently, the samples were blocked with 10% FBS (*v*/*v*) in 1× PBS for 30 min and then incubated overnight at 4°C with the corresponding primary antibodies given above in PBS/5% FBS: anti-AT1R (extracellular) antibody, anti-AT2R antibody, anti-AT4R antibody (IRAP), and anti-MasR. The primary antibody was removed from the samples, which were then washed three times in 1× PBS for 5 min each. The samples were incubated with the secondary anti-IgG antibody Alexa Fluor® 488 (rabbit polyclonal goat IgG secondary antibody conjugated to Alexa Fluor® 488; A11008; Invitrogen) in the dark for 1 h at room temperature. After that, the samples were washed again three times for 5 min with 1× PBS. Hoechst 33258 (Sigma Aldrich) was added at a concentration of 5 μg ml⁻¹ and incubated for 2 min.

The Alexa Fluor® 488 fluorochrome has maximum excitation and absorption wavelengths of 495 nm and 519 nm, respectively. Thus, the samples were excited with an argon laser at 488 nm and light was collected between 505 nm and 520 nm. Hoechst 33258 is a DNA marker that is cell membrane permeable and fluoresces in blue when it binds to the minor groove of the DNA double strand. Hoechst 33 258 has maximum excitation and absorption wavelengths of 352 nm and 461 nm, respectively, and its combination with Alexa Fluor® 488 fluorochrome is suitable because there is no overlap between their emission spectra.

Samples were prepared using Fluoromount G (EMS, Hatfield, UK) and observed under a confocal microscope (Fluoview FV 500; Olympus, Melville, NY, USA), allowing the overlap of images of consecutive sperm planes. The images were processed using Fluoview version 5.0 software, and image analysis was performed using ImageJ (developed on Mac OS X, the source code is freely available; National Institutes of Mental Health, Bethesda, MD, USA).

Statistics
We conducted descriptive statistical analysis of the data (mean, standard error mean [s.e.m.], median, and minimum and maximum values). These initial analyses provided information as a first approximation to begin the data analysis. Considering that the sample size was less than 50, this analysis was performed using the Shapiro–Wilk normality test. Statistical analysis of the data was performed using Microsoft Excel and the statistical package IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA). The Mann–Whitney U test was used to determine relationships between each of enzyme studied and sperm DNA fragmentation. Statistical significance and high statistical significance were determined by $P < 0.05$ and $P < 0.01$, respectively.

RESULTS
The highest levels of sperm peptide activity were observed for neutral aminopeptidase, followed by APB, and APA; the lowest value was for neutral endopeptidase (**Figure 1**). Similarly, the highest levels of peptide activity found in seminal plasma corresponded to APB and neutral aminopeptidase (with very similar values), followed by aspartyl aminopeptidase and prolyl endopeptidase (**Figure 1**). NEP in seminal plasma and PEP activity in spermatozoa were not found. Each enzyme was analyzed according to the value of fragmentation; ≤20% was considered non-fragmented, and >20% was considered fragmented. Higher mean values of peptidase unit activity were observed for APN, and minimum values for NEP. No differences were found between fragmented and non-fragmented spermatozoa.

The Mann–Whitney U test was used to determine the existence of a relationship existed between each of the studied enzymes and sperm DNA fragmentation. The maximum and minimum values, as well as the mean and s.e.m. with or without DNA fragmentation in sperm, are presented in **Figure 1**. The highest values in seminal plasma were observed for APN and APB; no differences depending on fragmentation status were found. Moreover, no association was found between the activity of the enzymes studied in seminal plasma in the presence or absence of sperm DNA fragmentation.

Enzymatic activity by flow cytometry
The percentage of APN/CD13 in non-fragmented DNA spermatozoa samples was higher than that in fragmented samples (**Figure 2a**). Furthermore, the intensity in non-fragmented was higher than that in fragmented spermatozoa ($P<0.05$; **Figure 2b**). The percentage of CD143 in non-fragmented samples was smaller than that in fragmented samples (**Figure 2a**), and the intensity in fragmented sperm was also higher than that in non-fragmented sperm (**Figure 2b**). The percentage of CD10 in non-fragmented DNA samples was smaller than that in fragmented DNA samples ($P<0.05$; **Figure 2a**), but the intensity in non-fragmented DNA samples was higher than that in fragmented DNA samples (**Figure 2b**).

Expression of receptors of the RAS: flow cytometry analysis
Levels of both AT1R and AT2R were higher in non-fragmented than in fragmented DNA spermatozoa. Moreover, the intensity in non-fragmented samples was higher than that in fragmented samples. The labeling of AT1R (94.3% in DNA fragmentation ≤20% *vs* 84.1% in DNA fragmentation >20%), AT2R (87.4% *vs* 63.1%) and PRR (96.4% *vs* 48.2%) were primarily expressed in non-DNA-fragmented spermatozoa (**Figure 3a**).

Regarding the presence of AT4R/IRAP, more than 90% of cells in both samples were positive for the receptor; though the intensity of non-fragmented DNA was 42.4 and that of fragmented was 21.3. The intensity percentages observed for PRR in non-fragmented DNA samples were higher than those in fragmented DNA (96.4% in DNA fragmentation ≤20% *vs* 48.2% in DNA fragmentation >20%). MasR was similar between both groups (97.4% *vs* 87.2%). PRR and MasR intensities in non-fragmented DNA spermatozoa were higher than those in DNA-fragmented spermatozoa ($P < 0.05$; **Figure 3b**).

The specificity of antibodies was determined by three different analyses:
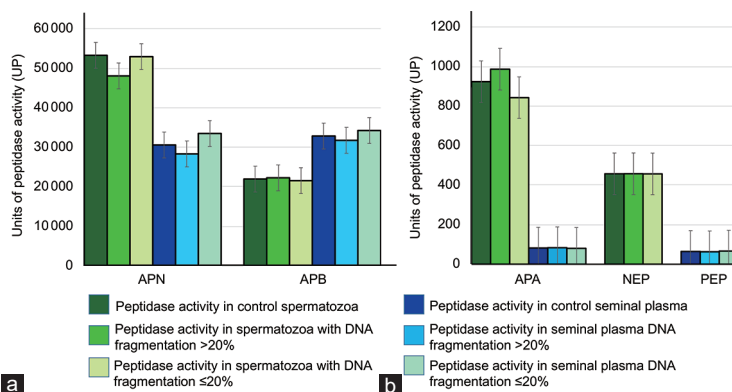1. Use of a sample to which primary and secondary antibodies were not added (blank)

Figure 1: Tested activities included (**a**) aminopeptidase N (APN/CD13; A III to A IV) and aminopeptidase B (APB; A III to A IV), (**b**) aminopeptidase A (APA; A II to A III), neutral endopeptidase (NEP; A I to A 1–7), and prolyl endopeptidase (PEP; which converts angiotensin [A] I and A II to A 1–7). NEP in seminal plasma and PEP activity in sperm were not found. A: angiotensin
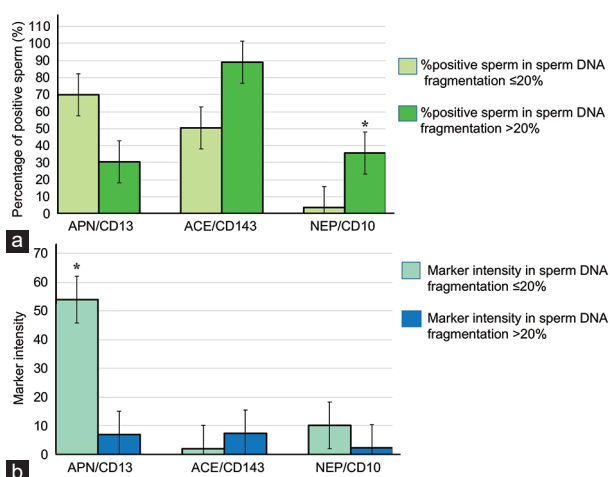

Figure 2: Presence of aminopeptidase N (APN/CD13), angiotensin-converting enzyme (ACE/CD143) and neutral endopeptidase (NEP/CD10), in spermatozoa with DNA fragmentation ≤20% and DNA fragmentation >20% by flow cytometry, shown as (**a**) percentage of positive sperm and (**b**) marker intensity. *$P<0.05$.

2. Addition of non-specific immunoglobulins at identical concentrations to those used with the primary antibodies (control of primary antibody)

3. Omission of the primary antibody before addition of the secondary antibody (control for secondary antibody). The observed fluorescence intensity patterns were very similar. Thus, the fluorescence measured in the samples was solely due to that emitted by the specific antibody binding to AT1R, AT2R, AT4R/IRAP, PRR or MasR (**Figure 3b**).

Expression of receptors of the RAS: immunocytochemical analysis
The AT1R receptor was detected in the sperm tail, and AT2R was localized in the equatorial/post-acrosomal region of the sperm head; a low-intensity signal of AT2R was also observed along the tail (**Figure 4**). In spermatozoa, PRR was found in the front of the sperm head, in the acrosomal region and at the back of the head; a weaker labeling was observed along the tail of the spermatozoon. In DNA-fragmented samples, however, the intensity was lower. In contrast, non-specific binding was not observed in negative controls in which the specific primary antibody was omitted.

Finally, no correlation was observed when the DNA fragmentation index values and analysis of RAS pathway components were performed per individual.

DISCUSSION

Peptidases are known to play a key role in growth control, differentiation, and the signal transduction in many cell systems by modulating the activity of bioactive peptides. In 2002, Fernández *et al*.[10] described for the first time the activity of several peptidases (enkephalin-degrading enzymes) in human seminal fractions. The high values observed in the different fractions (seminal fluid, prostasomes, cytosolic sperm fraction, and membrane fraction) suggested that both peptidases and their natural substrates might be involved in seminal physiology by regulating physiologically active peptides, not only in the testicle but also in the seminal fluid and spermatozoa.[10] Subsequently, these studies were expanded, demonstrating more specific roles for these peptide-regulating enzymes.[9,11,12] Thus, the regulatory enzymatic activities of peptide systems are involved in sperm fertilization processes, which represents a novel opportunity for reproductive management, by enhancing the probability of fertilization or reducing it through the development of novel targeted contraceptives.[11]

Over the years, studies have been extended to ascertain whether any of these peptide systems can be used as biomarkers of reproductive success in assisted reproduction processes, though none have been clearly described to date. In general, over 80 million people worldwide experience infertility and over one-third of infertility cases are due to male factors. Therefore, in addition to the study of opioidergic levels and their role in reproduction, a novel system has been analyzed in recent years to increase knowledge in reproductive function, to evaluate possible involvement in male reproductive pathologies and to identify biomarkers that might contribute to the development of therapeutic strategies for the treatment of male subfertility. We refer to the components of the RAS, peptide system known mainly for its importance in the maintenance of blood pressure as well as electrolyte and fluid homeostasis. Nonetheless, attention has also been paid to evidence of a widespread local RAS in several tissues regulating several specific functions. In this sense, this molecular system is also present in the reproductive tract and seems to act on male fertility by operating at multiple levels in the regulation of sperm fertilizing ability, suggesting its potential role as a therapeutic target for improving assisted reproduction therapies. Although the function of RAS in human spermatozoa is not completely understood, it would

be interesting to determine whether some RAS components can be considered biomarkers of sperm function and selection.

In fact, one of the components of this system has recently been described at the testicular level: the sperm protein tACE. The amount of this enzyme on sperm surface membranes suggests that it might play a role during embryo development, even before the activation of the translational machinery of the embryo. These results indicate that not only the presence or absence of sperm proteins but also the number of molecules per spermatozoon may provide very valuable information regarding embryo development, quality and viability. Thus, tACE may be useful as a biomarker to contribute information to embryologists in the selection of a sperm population with strong potential to produce high-quality embryos during ICSI.[20]

Accordingly, we report the levels of different enzymes in both sperm and seminal fluids that (in greater or lesser proportion) regulate levels of peptide components of RAS. Specifically, the enzymes evaluated were: PEP (A I and A II to A 1–7), NEP (A I to A 1–7), APN (A III to A IV), APA (A II to A III), APB (A III to A IV), and ACE (A I to A II). Because it is estimated that 10%–15% of men with sterility have gamete genetic material alterations, even though sperm concentration, mobility and morphology are normal, we also examined levels of these enzyme in sperm samples with DNA fragmentation to determine whether sperm DNA fragmentation is related to alterations in peptide metabolism and to describe biomarkers of sperm integrity and function, when comparing spermatozoa with and without DNA fragmentation.

Enzymes that regulate the levels of different RAS components (*e.g.*, regulatory aminopeptidases) tested in this study showed no significant alterations in activity between non-fragmented- and fragmented-DNA samples in seminal fluid or spermatozoa. This non-variation of seminal activity seems to demonstrate that, in this study, the seminal fluid that contains the gametes remains stable. However, at the cellular level, we observed differences in the APN/CD13 and ACE/CD143 levels by both immunofluorescence and flow cytometry. A III/A IV/AT4R pathway activity, regulated by APN, was reduced in DNA-fragmented spermatozoa, though in this case, there was no variation in expression of the receptor. It is difficult to interpret these results, because only the presence of APN has been previously described in spermatozoa, whereas A IV/AT4R has not yet been detected, and this is the first time that the presence of the AT4 receptor on sperm has been described. The A I/A II/AT2R axis regulated by ACE increases in fragmented samples due to receptor expression variation. It can therefore be deduced that this main RAS pathway is affected in spermatozoa with DNA fragmentation. Owing to the principal location of the enzymes and receptors altered in this principal axis of RAS (the tail of spermatozoa), it may be suggested that in cells with DNA fragmentation, sperm motility is more altered than the acrosome reaction of the spermatozoon.
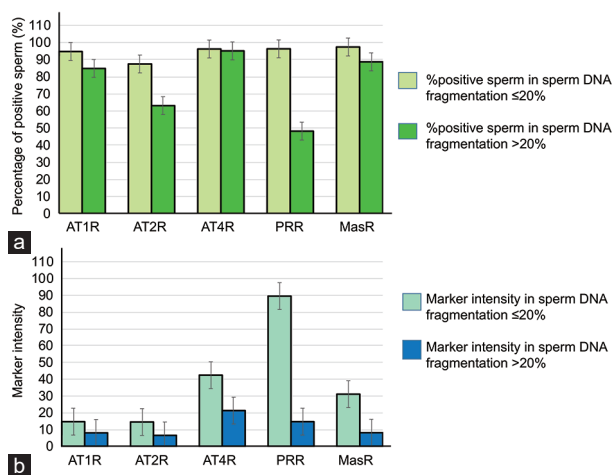
Figure 3: Presence and intensity of angiotensin (A) II type 1 receptor (AT1R), A II type 2 receptor (AT2R), A IV receptor (AT4R/IRAP), (pro)renin receptor (PRR) and A 1–7 receptor (MasR) in human spermatozoa with DNA fragmentation ≤20% and DNA fragmentation >20% by flow cytometry, shown as (**a**) percentage of positive sperm and (**b**) marker intensity.
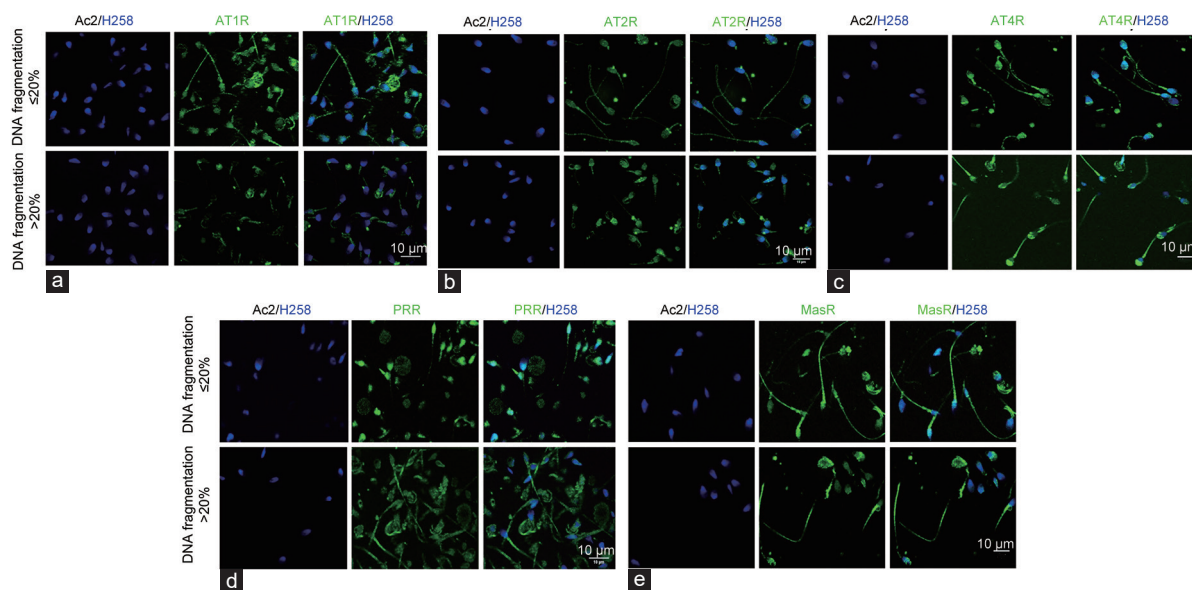
Figure 4: Results by immunocytochemistry of renin-angiotensin receptors in spermatozoa with DNA fragmentation ≤20% and with DNA fragmentation >20%: (**a**) angiotensin (A) II receptor type 1 (AT1R), (**b**) A II type 2 receptor (AT2R), (**c**) A IV receptor (AT4R/IRAP), (**d**) (pro)renin receptor (PRR), and (**e**) A 1–7 receptor or Mas receptor (MasR). H258: Hoechst 33258; Ac2: secondary antibody
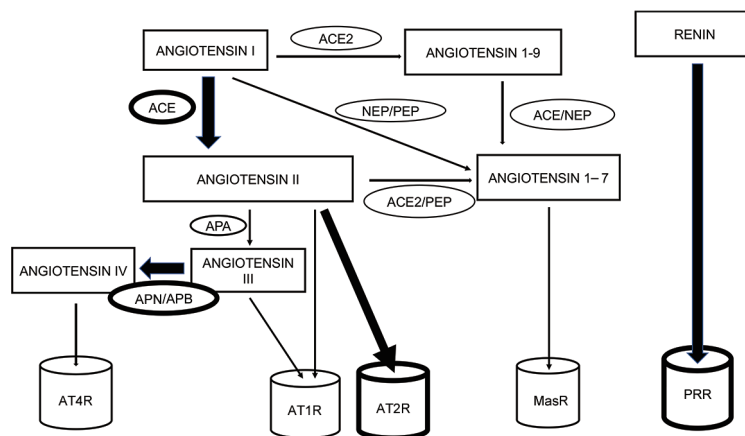
Figure 5: Schematic representation of the sperm renin angiotensin system, with the alterations (in bold) that occur in its axes in spermatozoa with DNA fragmentation. Due to the principal location of the enzymes and receptors altered in this principal axis of renin angiotensin system (RAS) (the tail of spermatozoa), it could be suggested that the spermatic motility is modified in DNA fragmented cells. ACE: angiotensin-converting enzyme; NEP: neutral endopeptidase; PEP: prolyl endopeptidase; MasR: angiotensin (A) 1–7 or Mas receptor; APN: aminopeptidase N; APA: aminopeptidase A; APB: aminopeptidase B; AT1R: A II receptor type 1; AT2R: A II receptor type 2; AT4R: A IV receptor; PRR: (pro)renin receptor.

ACE is present in mature spermatozoa of different species, and a recent study[32] have demonstrated the involvement of ACE in sperm motility, capacitation, the acrosome reaction and sperm-oocyte fusion. Thus, the reproductive capacity of fragmented sperm may be altered by this increase. ACE could thus be useful as a biomarker to aid embryologists in the selection of a sperm population. Additional studies should be carried out to conclude that it can also serve as a biomarker for DNA fragmentation. To facilitate the monitoring of our results, in **Figure 5**, we represent the local spermatozoon RAS axis and the variations that occur in fragmented spermatozoa.

AT1R was detected in the sperm tail, a finding that agrees with the report by Vinson *et al*.[33,34] Considering that the location of most RAS receptors (except perhaps PRR) is in the sperm tail, as suggested by Vinson *et al*.,[35] we infer that these receptors are related to the regulation of sperm mobility more than to the acrosome reaction. Considering the location of PRR, it might be involved in the acrosome reaction, a finding that coincides with that suggested by different authors regarding the relationship between this possibility and RAS.[36–41] In addition, as previously suggested, both renin precursor protein and the renin protein itself might act on PRR regardless of the classical axis.[42] Regarding our micrographs with RAS sperm receptors, we could not discriminate the immunolabeling pattern of non-fragmented and fragmented samples; that is, the location in the cell membrane persisted regardless of the sperm DNA fragmentation status. In fact, we observed that AT1R labeling, AT4R and MasR were similar between the sperm groups analyzed (with higher levels in sperm with non-fragmented DNA). Nevertheless, AT1R, AT2R and PRR showed higher expression in spermatozoa with fragmented DNA. In addition, the labeling intensity, which was lower in spermatozoa with fragmented DNA in all cases, should be considered. Regardless, it is impossible to determine whether the difference was due to the greater or lower number of receptors present or more spermatozoa expressing them. AT2R has already been described in human spermatozoa,[43] and it may also play an important role in sperm motility, as it has been observed that AT2R is related to human sperm concentration and motility.

The presence of PRR has recently been described,[32] suggesting that this receptor plays a role in sperm motility, as lighter staining was observed along the tail of human spermatozoa.

Finally, the fewer immunolabeled spermatozoa when DNA fragmentation was high and the intensity was low could be due to two circumstances: (1) spermatozoa with fragmented DNA have a lower responsiveness to active RAS peptides when they have a lower labeling intensity; or (2) fewer spermatozoa express the receptors, leading to lower immunolabeling intensity.

Although the latter possibility may seem the most logical, considering the results of this study and the literature, we suggest that the former is more likely. Thus, the receptors have a lower capacity to induce a response because there are fewer of them. This conclusion can be clearly inferred by comparing the percentage of gametes with and without DNA fragmentation that are positive for AT4R labeling. However, the intensity in spermatozoa with fragmented DNA was approximately half of that in spermatozoa with non-fragmented DNA. In conclusion, RAS is involved in the cellular response to sperm DNA fragmentation. Owing to the limitations of the study (*i.e.*, power analysis of 70%, and visual determination of the fragmentation degree), further studies are needed to determine the actual role of the RAS facility in sperm fragmentation processes and the possible validation of its components as biomarkers for reproductive success.

AUTHOR CONTRIBUTIONS

MVAP and LCS conceived of the study, designed the experiments, performed the statistical analysis, and wrote the manuscript. MVAP, MVRG and YFI carried out all the fertility procedures. AVP, GHB, and EEO conducted fluorometric, flow cytometry, and immunocytochemistry experiments. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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