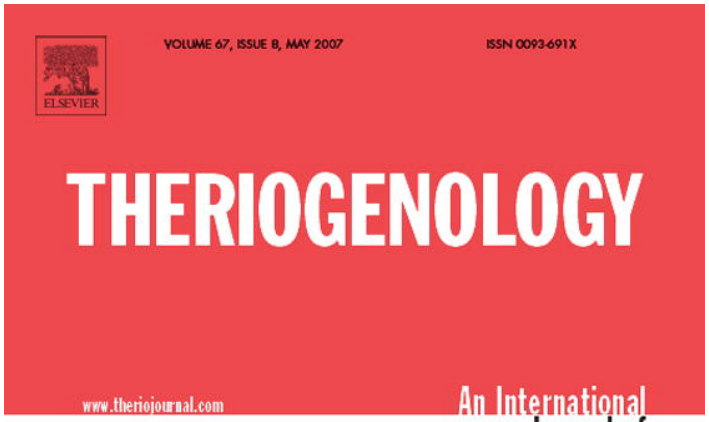

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Expression of the GM-CSF receptor in ovine spermatozoa: GM-CSF effect on sperm viability and motility of sperm subpopulations after the freezing–thawing process

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Abstract

The granulocyte–macrophage colony stimulating factor (GM-CSF) is a pleiotropic cytokine capable of stimulating proliferation, maturation and function of haematopoietic cells. Receptors for this cytokine are composed of two subunits, alpha and beta, and are expressed in myeloid progenitors and mature mononuclear phagocytes, monocytes, eosinophils and neutrophils, as well as in other non-haematopoietic cells. We have previously demonstrated that bull spermatozoa express functional GM-CSF receptors that signal for increased glucose and vitamin-C uptake and enhance several parameters of sperm motility in the presence of glucose or fructose substrates. In this study, we have analyzed the expression of GM-CSF receptors in ovine spermatozoa and studied the effect of GM-CSF on sperm viability and motility after the freezing–thawing process. Immunolocalization and immunoblotting analyses demonstrated that ovine spermatozoa (Xisqueta race) expressed GM-CSF receptors. In addition, GM-CSF partially counteracted the impairing action of freezing/thawing on the percentage of total motility, as well as on the specific motility patterns of each of the separate, motile sperm subpopulations of ram ejaculates subjected to this protocol. These results suggest that GM-CSF can play a role in the resistance of ram spermatozoa to environmental thermal stress.

Keywords: GM-CSF; Cryopreservation; Ovine spermatozoa; Motility; Sperm subpopulations

1. Introduction

We have recently demonstrated that bovine spermatozoa express functional low- and high-affinity GM-CSF receptors that signal for increased glucose and vitamin-C uptake [1]. GM-CSF is a glycoprotein with several molecular-weight species ranging from 18 kDa to over 30 kDa with hormone-like properties [2], capable of stimulating the proliferation of multipotential cells as well as inducing the proliferation, differentiation, maturation and functional activation of granulocytes and macrophages [3]. GM-CSF is expressed by haematopoietic, and also in several non-haematopoietic cell types such as osteoblast, smooth muscle, endothelial and epithelial cells [4], and murine foeto-placental tissue [5,6].

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The biological activity of GM-CSF is mediated through specific cell-surface receptors which consist of two interacting subunits, alpha and beta [7,8]. Receptors for GM-CSF are also present in non-haematopoietic cells such as placental trophoblasts, endothelial cells, oligodendrocytes of the central nervous system [9–12], follicular fluid and ovarian cells [13] and fallopian tubes [14]. Although there is evidence indicating that GM-CSF is expressed in some non-haematopoietic cells, the physiological role of GM-CSF in male reproductive tissues is unknown. To further analyze the role of GM-CSF in male germ cells, we have provided evidence that seminiferous tubule cells of human and bovine testes express GM-CSF, suggesting that this cytokine might have a potential role in the proliferation and differentiation process of spermatogenesis. We have also demonstrated that this growth factor plays a role in sperm motility and suggest that it may probably control sperm fertilizing ability [15,16].

It is herein reported that GM-CSF receptors are expressed in ovine spermatozoa. Furthermore, the presence of GM-CSF partially counteracted the impairing action of freezing/thawing on the percentage of total motility, as well as on the specific motility patterns of each of the separate, motile sperm subpopulations of ram ejaculates subjected to this protocol. These results seem to indicate that GM-CSF can play a physiological role in sperm as an agent which collaborates in sperm resistance to environmental stress, like that induced by extreme changes of temperature.

2. Materials and methods

2.1. Samples collection

Ovine ejaculated semen was collected with an artificial vagina from two 3-year-old sheep from the Centro de Inseminación Artificial of the Universidad Austral de Chile and from two adult (2–3 years old) Xisqueta rams housed at the experimental farm of the Universitat Autònoma of Barcelona. Xisqueta is a rustic, native sheep breed of Catalonia (Spain) in danger of extinction. Ejaculates were obtained twice a week from November 2004 to December 2004. After collection in the farm, the tubes were placed in a bath at 37 °C and taken to the laboratory. Concentrations of the samples were determined at 200× with either a Neubauer or Thoma haemocytometer cell chamber. Mass motility was examined at 40× and graded on a scale of 0–5 (0 = non-motile, 5 = dense semen with highly vigorous motility). Any sample with a mass motility below 3 was discarded. The ejaculate of each male was analyzed separately and an aliquot of each one was pooled and analyzed also. Motility, viability and membrane integrity were analyzed on each sample before further processing, applying the techniques described in Section 2.5.

2.2. Immunoprecipitation and immunoblotting

Spermatozoa were washed three times with a solution (pH 7.4) containing 150 mM NaCl, 10 mM sodium phosphate (PBS) and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4 °C. Cells were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulphate; SDS) containing several protease inhibitors (100 μg/mL PMSF, 2 μg/mL pepstatin A, 2 μg/mL leupeptin and 2 μg/mL aprotinin). The cell lysate was pre-cleared with Protein A–Sepharose CL-4B beads (Pharmacia; Uppsala, Sweden) for 60 min at 4 °C. For immunoprecipitation, equal amounts of protein (400 μg of total cell lysate) were incubated overnight at 4 °C with 2 μg of rabbit polyclonal antibodies of either the anti-alpha or anti-beta GM-CSF receptor (Santa Cruz Biotechnology; Sta. Cruz, CA, USA), followed by the addition of Protein A–Sepharose beads and incubated for a further 2 h at 4 °C. Bound immune complexes were washed three times with lysis buffer containing protease inhibitors and detergents. The pellet was eluted by boiling for 5 min with 2× Laemmli sample buffer [17]. Supernatant proteins were separated by SDS-PAGE as explained below, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation; Billerica, MA, USA), and immunoblotted with anti-alpha or anti-beta GM-CSF receptor subunits (1:500) (Santa Cruz Biotechnology; Sta. Cruz, CA, USA). Specific bands were visualized by ECL® (enhanced chemiluminescence; Amersham Biosciences; Arlington Heights, IL, USA) following [18].

2.3. Immunostaining procedures

Spermatozoa were washed three times with 1× PBS pH 7.4, 1 mM PMSF at 4 °C and incubated overnight at 4 °C with anti-alpha (1:100 dilution) or anti-beta (1:50 dilution) GM-CSF receptor subunits in 1% BSA–PBS pH 7.4 and 0.3% Triton X-100, followed by donkey anti-rabbit IgG-Alexa 488 (Invitrogen; Los Angeles, CA, USA). As controls, spermatozoa were incubated with antibodies pre-absorbed with the respective peptide used to generate the antibodies. Specifically,

the primary antibody was pre-incubated overnight at 4 °C with a 10-fold molar excess of synthetic peptide (Santa Cruz Biotechnology). Stained cells were examined with an Olympus Fluoview FV1000 laser-scanning confocal microscope. The images obtained were processed with Adobe Photoshop 6.0.

2.4. Semen freezing and thawing

Samples were divided into two equal parts: one treated with GM-CSF and the other one as a control. The extender utilized for cooling/freezing ram sperm was that which is described in [19]. This extender consisted of a Tris/HCl medium (3.64 g/L, pH 7.4) containing 0.5 g/L glucose, 1.99 g/L citric acid, 15% (v/v) egg yolk and 4% (v/v) glycerol. When stated, GM-CSF was added to the cooling/freezing extender to a final concentration of 5 nM. Samples were diluted in their respective extenders to a final concentration of 100×10^6 spz/mL, packed in 0.25-mL straws and then placed in a bath at 20 °C, which was kept in a cold room at 5 °C. This caused the temperature of the samples to decrease from 20 °C to 5 °C in 2 h. After this time, samples were frozen in liquid N_2 vapors by placing the straws 3 cm above the surface of a thermal-resistant recipient containing liquid N_2 for 8 min. Afterwards, straws were placed inside the liquid N_2 and they were further stored in liquid N_2 until their analysis. Frozen samples were thawed 2–3 weeks after freezing. Thawing of the samples was performed by placing the straws at 37 °C for 15 s, and analyses were performed immediately after thawing.

2.5. Assessment of ram-sperm quality

Percentages of viability, altered acrosomes and morphological abnormalities were determined by using the Eosin–Nigrosin staining procedure [20] after counting 200 spermatozoa per slice (1000× magnification-oil immersion). Following this stain, viable spermatozoa were defined as those that showed a uniform, white color under observation, whereas non-viable sperm were defined as those which showed any sign of both partial and total pinkish-purple staining. Additionally, sperm with intact acrosomes were defined as those that showed a uniform and continuous acrosomal ridge under the stain. All of the other acrosome aspects were considered to be spermatozoa with altered acrosomes. The functional integrity of the sperm membrane was evaluated using the hypoosmotic swelling test (HOS test; [21]). To perform this test, 100 μL of semen samples were added to 900 μL of a hypoosmotic solution containing 1.3% (w/v) fructose and 0.7% (w/v) dihydrate sodium citrate (osmolarity: 150 ± 4 mOsm) and incubated at 37 °C for 15 min. A negative control point of the HOS test was performed through the incubation of 100 μL of semen samples with 900 μL of an isoosmotic solution containing 3.7% (w/v) dihydrate sodium citrate in distilled water (osmolarity: 300 ± 6 mOsm) and also incubated at 37 °C for 15 min. After the incubations, a 30–40 μL aliquot of each hypo- and isoosmotic incubation was taken to perform an Eosin–Nigrosin staining as described above. The positive response to the test was quantified as the percentage of spermatozoa with swollen tails following the formula:

$$\mathrm{HOS}(\%) = \mathrm{SWH} - \mathrm{SWI},$$

where SWH is the percentage of swollen tails in the hypoosmotic medium and SWI is the percentage of swollen tails in the isoosmotic medium. The test was quantified after counting 200 spermatozoa per slide at 1000× magnification under an optical microscope.

Motion characteristics of the samples were evaluated by using computer-assisted analysis (CASA; Sperm Class Analyzer, Microptic; Barcelona, Spain). The CASA system used was based on the analysis of 16 consecutive, digitalized photographic images which were taken in a time-lapse of 0.64 s, which implied a velocity of image-capturing of one photograph every 40 ms. Images were taken from 5-μL drops of the samples, which were placed on pre-warmed slides at 37 °C and covered with 20-mm × 20-mm coverslips. From 3 to 5 randomly-chosen fields were taken for each sample at a magnification of 200× on a dark field. Under these conditions, total motility was defined as the percentage of spermatozoa per sample which showed a curvilinear velocity (VCL) >20 μm/s. All CASA-obtained motility parameters are shown in Table 1.

2.6. Statistical analyses

Statistical analyses were performed by using the SAS statistical package [22]. Data derived from percentages of viability, altered acrosomes, morphological abnormalities and the mean, overall CASA motility parameters of ram sperm were analyzed firstly in order to determine the normality of obtained data. Those that did not show a normal distribution were then transformed to normalization, by using either the appropriate logarithmic or angular transformation equations. After this, the normalized data were analyzed through a General Linear Model (PROC GLM) to evaluate significant differences ($P < 0.05$) among

Table 1
Definition of motility parameters obtained after CASA analysis

Parameter	Units	Description
Curvilinear velocity (VCL)	μm/s	The instantaneously recorded sequential progression along the entire trajectory of the spermatozoon
Linear velocity (VSL)	μm/s	The straight trajectory of the spermatozoon per unit of time
Mean velocity (VAP)	μm/s	The mean trajectory of the spermatozoon per unit of time
Linear coefficient (LIN)	%	VSL/VCL
Straightness coefficient (STR)	%	VSL/VAP
Wobble coefficient (WOB)	%	VAP/VCL
Mean lateral head displacement (mean ALH)	μm	Mean head displacement along its curvilinear trajectory around the mean trajectory
Maximal lateral head displacement (max ALH)	μm	Maximal head displacement along its curvilinear trajectory around the mean trajectory
Dance (DNC)	$\mu m^2/s$	VCL × mean ALH
Mean dance (DNM)	μm	Mean ALH/LIN
Angularity index (AI)	%	The percent value of the angle formed by two successive trajectories
Angular velocity (AV)	μm/s	(VCL × AI)/100
Absolute angular mean displacement (AbsMAD)	Angular degrees	The absolute value of the advancing angle of the sperm trajectory
Algebraic angular mean displacement (AlgMAD)	Angular degrees	The algebraic value of the advancing angle of the sperm trajectory. Negative values indicate a clockwise displacement
Frequency of head displacement (BCF)	Hz	The number of lateral oscillatory movements of the sperm head around the mean trajectory
Minor harmonic oscillation of the head (HLO)	μm	The minimum value of the distance between the curvilinear trajectory, with respect to the mean trajectory
Major harmonic oscillation of the head (HHI)	μm	The maximal value of the distance between the curvilinear trajectory, with respect to the mean trajectory
Mean harmonic oscillation of the head (HME)	μm	The mean value of the distance between the curvilinear trajectory, with respect to the mean trajectory
Maximal amplitude of the oscillation of the head (HMX)	μm	Maximal distance between two successive crosses around the mean trajectory
Basic harmonic oscillation of the head (HBS)	μm	Mean distance between two successive crosses around the mean trajectory
Harmonic amplitude (H_Y)	μm	Minimal distance between two successive crosses around the mean trajectory

groups. Furthermore, the LSMEANS procedure was used to compare the results obtained among them. The combination of both of these procedures yielded the results described in Section 3. It must be stated that the data shown in tables and figures are those obtained before normalization, although the appropriate statistical analyses were performed on normalized data. Additionally, further statistical analysis was performed in order to establish if the addition of GM-SCF affects the specific, motile sperm-subpopulations structure of ram sperm after freezing/thawing. This analysis was performed following a chronological development. Thus, the first question was to evaluate which of the motion parameters obtained after the CASA analysis were truly significant in the interpretation of the data under the subpopulations-structure point of view. In this sense, it must be remembered that our CASA system produces 21 separate motion parameters, many of which are closely related to each other. In this sense, it was necessary to reduce the number of variables with the purpose of selecting parameters which would explain the overall sperm movement more accurately. To evaluate this, the sperm motility descriptors obtained from CASA were clustered into separate variable groups by the VARCLUS procedure. These analyses grouped the tested motion parameters into four separate clusters (data not shown). Following the results of the VARCLUS procedure, the choice of the individual parameter that, within each cluster, conserved the maximal information over the total parameters was made by studying each one's values of R^2 with its own cluster and, if necessary, its values of R^2 with the next closest cluster. Further analysis of the relationship among parameters in each cluster led to the choice of the individual parameter that, within each cluster, conserved the maximum information regarding total parameters. Following this analysis, the chosen parameters were:

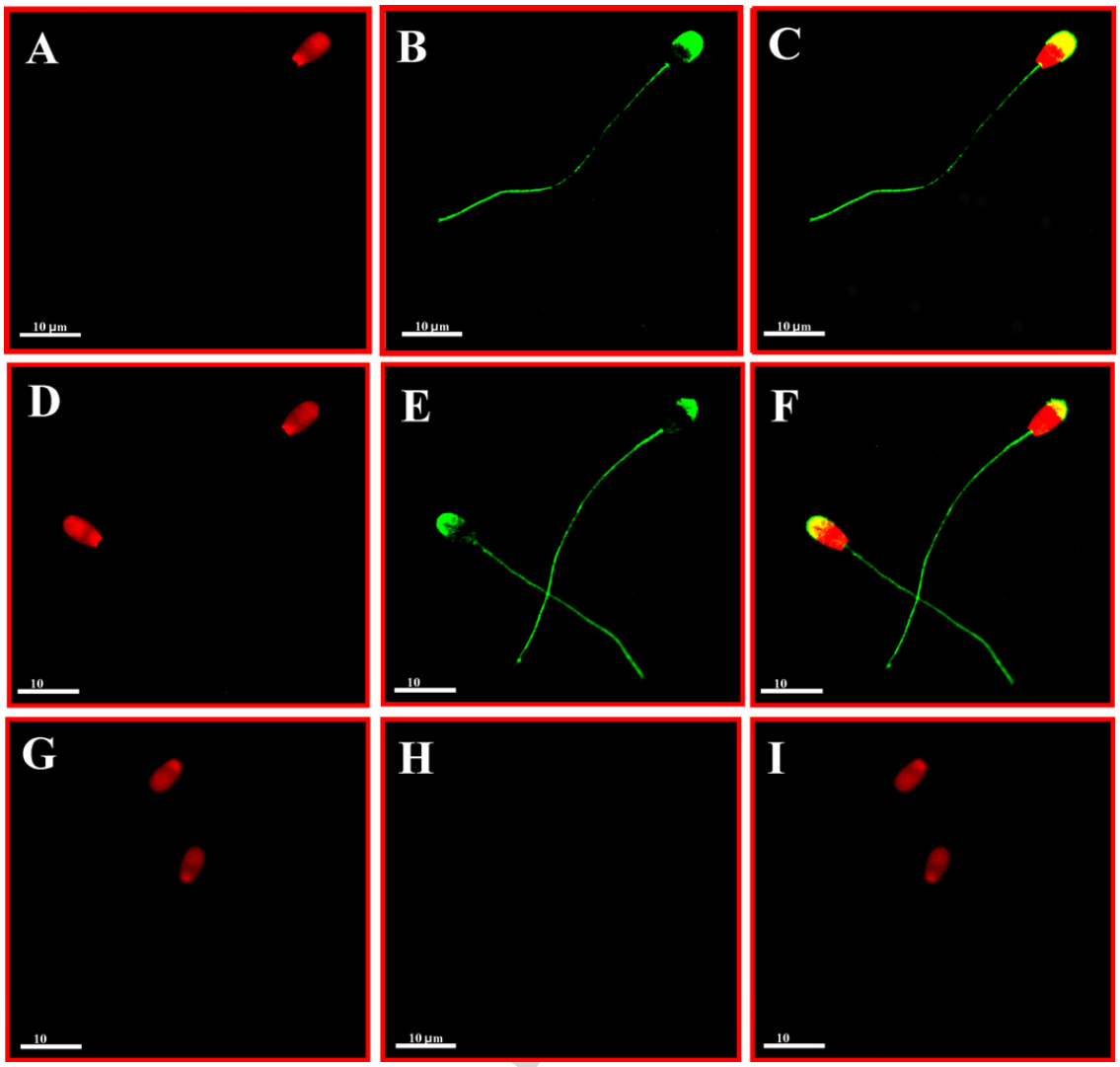

Fig. 1. Immunolocalization of GM-CSF receptors in ovine spermatozoa. For immunofluorescence, ovine spermatozoa were spread onto coated slides and reacted with anti-alpha (B and C) and anti-beta (E and F) GM-CSF receptor antibodies and visualized using anti-rabbit IgG-Alexa 488 secondary antibodies (green). Cells incubated with preadsorbed antibodies did not show a positive reaction (H and I). Nuclei were counterstained with propidium iodide (red), and confocal images were acquired. (A, D, G) Images showing only the nuclear, propidium iodide stain. These are representative images from eight independent determinations. Scale bars, 10 μm.

Linear coefficient (LIN): The coefficient percent between linear velocity and curvilinear velocity. Units are in %.
Wobble coefficient (WOB): The coefficient percent between mean velocity and curvilinear velocity. Units are in %.
Dance (DNC): The result of the multiplication of curvilinear velocity and mean lateral head displacement. Units are in $\mu m^2/s$.
Lateral head displacement (HME): The mean value of the distance between the curvilinear trajectory, with respect to the mean trajectory. Units are in μm.

The next step was the analysis of the presence of concrete, motile sperm subpopulations in the ejaculates after analyzing sperm motility using the CASA motion parameters selected by the VARCLUS procedure as a basis. For this purpose, another clustering procedure, FASTCLUS, was used [23,24]. The FASTCLUS procedure performs a disjointed cluster analysis on the basis of Euclidean distances computed from one or more quantitative variables, in this case, the selected motility parameters. This permits the study of large sets of observations, from approximately 100–100,000, which is ideal for analyzing the spermatozoa observed in our study. In this way, FASTCLUS divided the spermatozoa into clusters such that every cell belonged to one and only one cluster. Spermatozoa that were very close to each other were assigned to the same cluster, while spermatozoa which were far apart were in

different clusters. The separation of spermatozoa into clusters was performed on the total number of spermatozoa obtained in the diluted semen samples. Globally, 10,690 motile sperm were analyzed, from which 965 were from fresh samples, 4752 were from frozen/thawed, control samples and 4973 were from frozen/thawed samples with GM-CSF added.

The FASTCLUS procedure divides the motile sperm population in the whole of the studied samples into clusters. However, a more in-depth study was needed to determine the specific differences among subpopulations, and how this structure varies in the separate conditions is studied in this work. For this purpose, a General Linear Model (PROC GLM) was again used to evaluate significant differences ($P < 0.05$) among clusters of sperm subpopulations, whereas the LSMEANS procedure was used to compare the obtained sperm subpopulations among them. The combination of both of these procedures gave the results described in Section 3.

3. Results

3.1. Detection of the GM-CSF receptor in ram spermatozoa

The detection of the GM-CSF receptor in ovine spermatozoa was assessed by immunocytochemistry using the GM-CSF antibody directed against proteins reactive with anti-human alpha- and beta-subunit antibodies (Fig. 1). Detailed immunofluorescence analysis indicated that ovine spermatozoa were immunoreactive with both alpha- and beta-antibodies, with the immunoreactivity specifically associated with the sperm tail and acrosome for the alpha-subunit (Fig. 1A–C) and beta-subunit in fresh, ejaculated spermatozoa (Fig. 1A–C). Similarly to that observed in human and bovine spermatozoa, there was always a greater intensity of staining with the alpha-antibody as compared with the beta-antibody in ovine spermatozoa [1,15,16]. The immunoreactive signal and the localization pattern for both subunits of the GM-CSF receptor in frozen/thawed spermatozoa in the absence or presence of GM-CSF was similar to fresh spermatozoa (data not shown). Negative results were obtained when cells were incubated with pre-absorbed antibodies with the corresponding peptide for GM-CSF (Fig. 1G–I).

Confirming the results of the immunolocalization studies, immunoblotting of membrane proteins extracted from ovine spermatozoa and immunoprecipitated with alpha and beta GM-CSF receptor antibodies demonstrated the presence of immunoreactive protein bands. The anti-alpha antibody reacted with a protein band with an apparent MW of 94 kDa (Fig. 2A). On the other hand, the anti-beta antibody reacted with a main band with an apparent MW of 120 kDa (Fig. 2B).

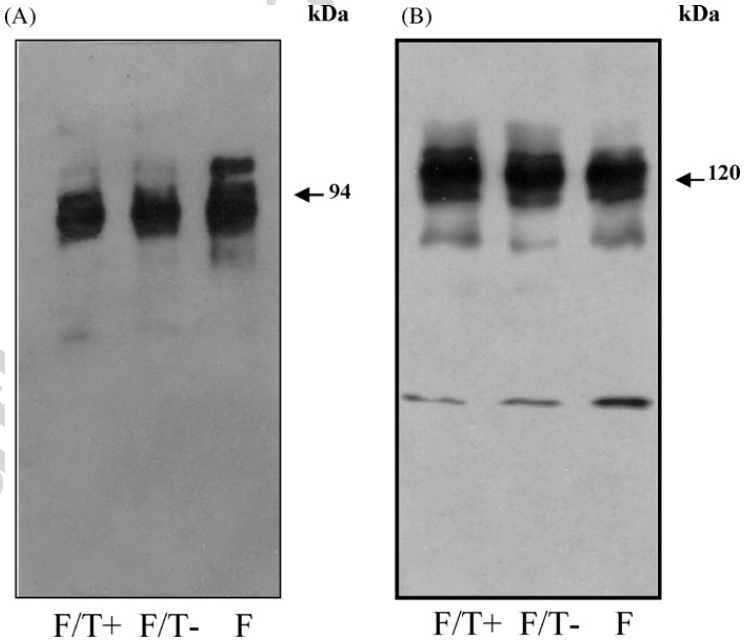

Fig. 2. Identification of GM-CSF receptors in fresh and frozen/thawed ovine semen. Total proteins isolated from fresh ovine spermatozoa (F) and spermatozoa subjected to the freezing/thawing process in the presence (F/T+) or absence of GM-CSF (F/T−) were immunoprecipitated with anti-GM-CSF receptor antibodies and resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, transferred to PVDF membranes, and probed with anti-alpha (A) and anti-beta (B) subunit antibodies, followed by incubation with a secondary antibody coupled to peroxidase. ECL sizes on the right are in kDa and indicate the migration of molecular mass standards.

Table 2
Percentages of viability, altered acrosomes, HOS test and total motility in fresh ram spermatozoa and frozen/thawed samples in the presence and the absence of GM-CSF

Parameters	Fresh samples	Frozen/thawed samples without GM-CSF	Frozen/thawed samples with GM-CSF
Viability (%)	58.0 ± 1.5^{a}	19.7 ± 1.2^{b}	21.6 ± 1.2^{b}
Altered acrosomes (%)	43.4 ± 1.2^{a}	81.2 ± 3.6^{b}	78.0 ± 2.7^{b}
HOS (%)	31.5 ± 1.0^{a}	5.8 ± 0.8^{b}	6.7 ± 0.8^{b}
Total motility (%)	88.9 ± 0.4^{a}	55.2 ± 0.3^{a}	67.3 ± 0.3^{c}

The percentages of viability, altered acrosomes, HOS test and total motility have been defined in Section 2. Values are expressed as mean ± S.E.M. of nine different experiments. Different superscripts in a row indicate significant ($P < 0.05$) differences among data.

There were no differences in the amount of alpha- and beta-subunits detected in proteins extracted from fresh, ejaculated spermatozoa compared to those frozen/thawed, either in the presence or in the absence of GM-CSF (Fig. 2A and B, lanes 1–3). For the alpha-subunit, however, there is a higher signal in proteins extracted from fresh, ejaculated spermatozoa (Fig. 2A, lane 3). This result is consistent with the observed results in the localization studies (Fig. 1B).

3.2. Effects of GM-CSF on the overall semen quality of ram sperm after freezing/thawing

Freezing/thawing had a profound effect on the overall semen quality of ram sperm, evaluated through the determination of the percentages of viability, altered acrosomes, total motility and the HOS test. Thus, all of these parameters underwent an intense and significant ($P < 0.05$) decrease after freezing/thawing (Table 2). The addition of GM-CSF to the freezing extender to a final concentration of 5 nM did not significantly modify the evaluated semen-quality parameters, excepting the percentage of total motility after the CASA analysis, which rose from about 55% in untreated frozen/thawed samples to about 60% in cells with the effector added (Table 2).

3.3. Effects of GM-CSF on the motility parameters and motile sperm subpopulations structure of ram semen after freezing/thawing

The addition of GM-CSF also affected some of the mean motility parameters of ram sperm after freezing/thawing. In this way, values of mean ALH were significantly ($P < 0.05$) lower in GM-CSF-added thawed cells than in the control ones, whereas the absMAD and HHI were significantly ($P < 0.05$) greater (Table 3). Notwithstanding, the study of the mean values of motility parameters in an ejaculate does not allow for an exact description of the putative effects of any effector on motility, since this analysis dismisses the importance of changes in the specific motile sperm subpopulations structure in an ejaculate, which could vary without a great effect on the mean, overall values of motility. In this way, the study of the motile sperm subpopulational structure of ram ejaculates showed that this consisted of a 4-subpopulations one. These subpopulations can be characterized as follows (see Table 4):

Subpopulation 1: This subpopulation was made up of sperm with high linearity and "strength" in their movement, as shown by the values of LIN, WOB and DNC. Moreover, they showed relatively low values of head oscillation, as indicated by the HME. This was the major population in the samples, reaching values of 39.5% of the total motile subpopulation in fresh samples (Table 4).
Subpopulation 2: This subpopulation was made up of sperm with mild-to-high linearity and "strength" in their movement, as shown by the values of LIN, WOB and DNC. Furthermore, these spermatozoa showed high values of head oscillation, as indicated by the HME, specially when compared with Subpopulation 1. Subpopulation 2 included 27.3% of the total motile subpopulation in fresh samples (Tables 4 and 5).
Subpopulation 3: This subpopulation showed a mean linearity and a relatively high "strength", as shown by the values of LIN, WOB and DNC. Moreover, this subpopulation showed relatively high values of head oscillation, as indicated by the HME. Subpopulation 3 reached values of 22.1% of the total motile subpopulation in fresh samples (Tables 4 and 5).
Subpopulation 4: This subpopulation combined a low linearity with a relatively high "strength" of its motion characteristics, as indicated by the values of LIN, WOB and DNC. Furthermore, values of HME were the lowest, thus indicating low head movement. In addition to this, Subpopulation 4 was the smallest population in the samples, reaching values of only 11.2% of the total motile subpopulation in fresh samples (Tables 4 and 5).

Table 3
CASA-obtained, mean values of motility parameters from fresh ram spermatozoa and from frozen/thawed samples in the presence and the absence of GM-CSF

Motility parameter	Fresh samples	Frozen/thawed samples without GM-CSF	Frozen/thawed samples with GM-CSF
VCL (μm/s)	83.1 ± 1.2^{a}	53.4 ± 0.4^{b}	52.7 ± 0.4^{b}
VSL (μm/s)	56.6 ± 1.1^{a}	18.2 ± 0.2^{b}	18.1 ± 0.2^{b}
VAP (μm/s)	62.8 ± 1.1^{a}	27.3 ± 0.3^{b}	27.0 ± 0.2^{b}
LIN (%)	64.7 ± 0.8^{a}	32.2 ± 0.3^{b}	32.4 ± 0.3^{b}
STR (%)	83.8 ± 0.7^{a}	58.5 ± 0.4^{b}	59.0 ± 0.4^{b}
WOB (%)	73.4 ± 0.6^{a}	50.2 ± 0.3^{b}	50.1 ± 0.2^{b}
Mean ALH (μm)	2.96 ± 0.05^{a}	2.15 ± 0.08^{b}	2.51 ± 0.02^{c}
Max ALH (μm)	7.25 ± 0.15^{a}	6.51 ± 0.06^{b}	6.33 ± 0.06^{b}
DNC ($\mu m^2/s$)	289.8 ± 0.3^{a}	175.6 ± 3.5^{b}	168.0 ± 3.4^{b}
DNM (μm)	0.08 ± 0.01^{a}	0.16 ± 0.01^{b}	0.15 ± 0.01^{b}
AI (%)	61.1 ± 0.6^{a}	43.3 ± 0.2^{b}	43.4 ± 0.2^{b}
AV (μm/s)	52.3 ± 1.0^{a}	23.5 ± 0.2^{b}	23.3 ± 0.2^{b}
AbsMAD (angular degrees)	52.1 ± 0.8^{a}	77.9 ± 0.3^{b}	70.4 ± 0.3^{c}
AlgMAD (angular degrees)	-6.8 ± 0.7^{a}	-19.5 ± 0.5^{b}	-19.0 ± 0.4^{b}
BCF (Hz)	13.8 ± 0.2^{a}	16.2 ± 0.1^{b}	16.2 ± 0.1^{b}
HLO (μm)	0.25 ± 0.02^{a}	0.08 ± 0.01^{b}	0.08 ± 0.01^{b}
HHI (μm)	2.71 ± 0.06^{a}	1.84 ± 0.02^{b}	1.99 ± 0.02^{c}
HME (μm)	1.21 ± 0.03^{a}	0.67 ± 0.01^{b}	0.68 ± 0.01^{b}
HMX (μm)	3.05 ± 0.05^{a}	2.05 ± 0.02^{b}	1.96 ± 0.02^{b}
HBS (μm)	22.0 ± 0.4^{a}	13.8 ± 0.2^{b}	13.4 ± 0.1^{b}
H_Y (μm)	0.69 ± 0.02^{a}	0.22 ± 0.01^{b}	0.21 ± 0.01^{b}

Sperm motility parameters have been defined in Table 1, whereas CASA analysis has been performed as described in Section 2. Values are expressed as mean ± S.E.M. of nine different experiments. Different superscripts in a row indicate significant ($P < 0.05$) differences among data.

Under the subpopulations structure point of view, freezing/thawing of samples without GM-CSF showed a steady decrease in the values of all of the motion parameters tested in all four subpopulations, excepting that of DNC of Subpopulation 4 (Table 4). The addition of GM-CSF to the freezing medium partially counteracted this overall decrease. In this sense, values of WOB in all four subpopulations were significantly ($P < 0.05$) greater in the frozen/thawed samples with the effector added than in those frozen without it (Table 4). Similarly, values of LIN of Subpopulations 2 and 3 were also significantly ($P < 0.05$) greater after freezing/thawing in the presence of GM-CSF (Table 4). Finally, freezing/thawing changed the mean percentage of each subpopulation in the total motile-sperm population of samples. Hence, Subpopulation 1 decreased from 39.5% of total motile sperm in fresh samples to 20.7% in control, frozen semen (Table 5). This percent decrease of Subpopulation 1 was accompanied by a proportional increase in the percentages of all of the other subpopulations, specially that of Subpopulations 3 and 4, which increased from 22.1% to 30.7% and from 11.2% to 19.1% after freezing/thawing, respectively (Table 5). The addition of GM-CSF did not significantly affect the freezing/thawing-induced changes on the percent structure of motile subpopulations (Table 5).

4. Discussion

The physiological role of GM-CSF receptors in non-haematopoietic tissues, especially in male germ cells, is still unclear. Herein is reported the presence of GM-CSF receptors in ram spermatozoa demonstrated by immunolocalization, Western blot analyses and functional assays. The results of the immunolocalization and immunoblotting experiments, using anti-alpha and anti-beta antibodies, support the notion that the ovine spermatozoa GM-CSF receptor has a subunit structure similar to the GM-CSF receptor present in haematopoietic cells for which the alpha- and beta-subunits have been characterized in detail [3]. In a previous study, we demonstrated that the GM-CSF receptor of human and bovine ejaculated spermatozoa displays a localization throughout the midpiece and the tail of spermatozoa [1], and we suggested a metabolic role of GM-CSF in the sperm cell. We also demonstrated that bovine spermatozoa express functional GM-CSF receptors assessed by increased glucose and the oxidized form of vitamin-C uptake via facilitative hexose transporters. We expected that facilitative hexose transport by GLUTs, as well as their interaction with the active GM-CSF receptors, might be related to the quality of the sperm's movement and found that the addition of 2 nM of GM-CSF

Table 4
Ram-sperm motile subpopulations structure in fresh ejaculates and in frozen/thawed samples in the presence and the absence of GM-CSF

Motility descriptors	Subpopulation 1			Subpopulation 2			Subpopulation 3			Subpopulation 4		
	Fresh	CTH	FTH	Fresh	CTH	FTH	Fresh	CTH	FTH	Fresh	CTH	FTH
LIN (%)	84.9 ± 0.6^{a}	63.7 ± 0.4^{b}	65.3 ± 0.4^{b}	66.3 ± 0.7^{a}	44.0 ± 0.3^{b}	56.2 ± 0.3^{c}	49.0 ± 0.8^{a}	29.5 ± 0.3^{b}	41.2 ± 0.3^{c}	24.4 ± 1.1^{a}	19.8 ± 0.4^{b}	20.5 ± 0.3^{b}
WOB (%)	$83.4 \pm 0.^{2a}$	76.3 ± 0.1^{b}	79.6 ± 0.1^{c}	75.5 ± 0.2^{a}	65.6 ± 0.1^{b}	69.5 ± 0.1^{c}	66.2 ± 0.2^{a}	55.4 ± 0.1^{b}	58.4 ± 0.1^{c}	50.4 ± 0.3^{a}	44.6 ± 0.1^{b}	47.4 ± 0.1^{c}
DNC ($\mu m^2/s$)	285.8 ± 2.7^{a}	264.7 ± 1.8^{b}	258.8 ± 1.8^{b}	302.2 ± 3.3^{a}	265.1 ± 1.5^{b}	269.7 ± 1.4^{b}	302.6 ± 3.5^{a}	246.9 ± 1.4^{b}	242.5 ± 1.4^{b}	252.6 ± 4.9^{a}	252.8 ± 1.8^{a}	251.6 ± 1.6^{a}
HME (μm)	1.17 ± 0.02^{a}	1.06 ± 0.01^{b}	1.04 ± 0.01^{b}	1.36 ± 0.02^{a}	1.02 ± 0.01^{b}	1.07 ± 0.01^{b}	1.37 ± 0.03^{a}	0.75 ± 0.01^{b}	0.79 ± 0.01^{b}	0.76 ± 0.04^{a}	0.56 ± 0.01^{b}	0.61 ± 0.01^{b}

Sperm motility parameters have been defined in Table 1, whereas CASA analysis has been performed as described in Section 2. Values are expressed as mean ± S.E.M. of nine different experiments. The total number of motile spermatozoa utilized for the analysis was 10,690, of which 965 were from fresh samples, 4752 were from frozen/thawed, control semen and 4973 were from frozen/thawed sperm added with GM-CSF. Different superscripts in a row from the same subpopulation indicate significant ($P < 0.05$) differences among data. Fresh: Data from fresh ram ejaculates. CTH: Frozen/thawed sperm without GM-CSF. FTH: Frozen/thawed sperm with GM-CSF.

increased several parameters of seminal motility [15,16].

The fact is noteworthy that freezing/thawing of ram spermatozoa caused a decrease in the alpha-subunit content of the GM-CSF receptor, whereas the expression of the beta-subunit was not modified in the same conditions. This result could be a consequence of the freezing/thawing-induced loss of some sperm structures. In this sense, it must be remembered that freezing/thawing induces a great decrease in the percentage of spermatozoa with intact acrosomes, which indicates that this procedure induces a destructuring and concomitant loss of the acrosome-forming structures. Following the described results regarding the immunolocalization of both alpha- and beta-subunits of the GM-CSF receptors, its would be logical to assume that the alpha-subunit would be, in the sperm head, strongly associated with some acrosome structure, which is lost during the freezing/thawing process. This hypothesis has to be confirmed by other experiments, i.e., immunogold localization and, in the event of being confirmed, this would indicate a separate localization of both subunits in the sperm head, this separation being an efficient mechanism to regulate GM-CSF receptor activity.

In the present study, the effects of GM-CSF in the modulation of ram-sperm resistance to freezing/thawing were advanced and tested. Our results seem to indicate that this effector could play some role in the resistance ability of this sperm. It is doubtless that data showed a small, protective effect of GM-CSF against freezing/thawing, and the majority of this effect was centered in a partial recovery of some motility parameters, as well as of the total motility percentage. The lack of a stronger effect could be due to the utilization of an inappropriate final GM-CSF concentration. Nevertheless, it must be stressed that the GM-CSF effect seems not to be uniform in all of the sperm of a single sample, since our results indicate the existence of separate, specific actions of the effector on the separate motile subpopulations in a single sample. These separate effects can pass unnoticed if we take and analyze the data obtained considering a homogenous sample, since the GM-CSF in a single subpopulation can be numerically counteracted by its effect, or lack of effect, on another subpopulation. Thus, although the mean data seem to indicate that GM-CSF has no clear effect on sperm function, the analysis based upon a subpopulations-based sample structure highlights the presence of a significant recovering action linked to GM-CSF, which was more or less intense depending on the specific subpopulation group that is analyzed. This supports the hypothesis that sperm included in separate

Table 5
Supopulation percentages in fresh, control frozen–thawed (CTH) and GM-CSF-treated frozen–thawed (FTH) ram semen

Subpopulation	Fresh	CTH	FTH
1	39.5[a]	20.7[b]	17.0[b]
2	27.3[a]	29.3[b]	29.7[b]
3	22.1[a]	30.7[b]	32.7[b]
4	11.2[a]	19.1[b]	20.6[b]

Results are shown as percentages of the total number of spermatozoa analyzed after nine different experiments (10,690 motile sperm, from which 965 were from fresh samples, 4752 were from frozen/thawed, control samples and 4973 were from frozen/thawed cells added with GM-CSF). Different superscripts in a row from the same subpopulation indicate significant ($P < 0.05$) differences among data after a Chi square Test. Fresh: Data from fresh ram ejaculates. CTH: Frozen/thawed sperm without GM-CSF. FTH: Frozen/thawed sperm with GM-CSF.

subpopulations have, in fact, different function levels, as the specific motility patterns indicate. As a consequence, the response to GM-CSF would differ among spermatozoa following their adscription to a separate subpopulation and, hence, to the specific sperm-cell function status.

It is also noteworthy that the observed effect of GM-CSF on frozen/thawed ram sperm was centered on motility. Sperm motility is modulated to a great extent by changes in the sperm's energy-obtaining mechanisms [25]. This is logical, since as much as 60% of the energy consumed by spermatozoa is devoted to maintaining motility [25]. Mammalian sperm obtains its energy mainly through both glycolysis and the Krebs cycle [25], and the precise equilibrium between both energy sources varies depending on the precise functional status of the cells. In this sense, whereas it has been indicated that the Krebs cycle-originated energy is needed to maintain motility in fresh sperm of several species [26–30], the same energy reaches only 5% of the total energy output in fresh boar-sperm [31]. On the other hand, functional changes during sperm lifetime, like those associated with capacitation and acrosome reaction, are strongly linked to coordinated changes in the Krebs cycle-obtained energy [32]. Taking into account all of these data, we can hypothesize that the observed effects of GM-CSF on sperm motility after freezing/thawing could be a consequence of some action of the effector on the sperm energy-supply mechanisms, specially those involving the Krebs cycle. A putative, protective action of mitochondrial function could thus aid in explaining the limited actions of GM-CSF on motility, especially taking into account the lack of effects on other sperm-function aspects that are not directly related to mitochondria, like the cytoplasmic-related parameters viability and HOS test as well as acrosome integrity. Of course, more experiments are needed, at different concentrations of GM-CSF, in order to elucidate this hypothesis. This will undoubtedly serve, under a practical point of view, to optimize the utilization of GM-CSF as a protective factor for mammalian sperm in stress conditions like freezing/thawing.

Our results showed a striking difference among percent values of parameters obtained by the Eosin–Nigrosin vital stain and those obtained through both CASA and the HOS Test. We think that the difference between the results of these parameters lies in that they are measuring sperm characteristics that are only loosely related themselves. Thus, the determination of viability and altered acrosomes through the Eosin–Nigrosin technique only reflects a punctual, passive aspect on sperm function, since it reflects a static situation that does not reflect the ability of sperm to react against media. Furthermore, the Eosin–Nigrosin technique does not reflect any membrane alteration that can be directly related to sperm motility. Thus, although a relationship between membrane integrity as reflected by Eosin–Nigrosin technique is totally logical, this does not imply that this relationship is direct, especially taking into account that Eosin–Nigrosin is not a very specific technique. Regarding the HOS Test, we must indicate that this test is a dynamic one, one that reflects the sperm's ability to react against an osmotic change of medium, and this ability does not have to be directly related to the initial membrane integrity as reflected by the Eosin–Nigrosin technique.

Regarding the existence of a specific motile-sperm subpopulations structure of ram ejaculates, it must be indicated that a similar structure, composed of three or four separate subpopulations, has already been described in a steadily increasing number of mammalian species, such as boar [24,33,34], gazelle [34], common marmoset [33], golden hamster [33], horse [23], deer [35] and donkey [36]. It is not surprising, then, that ram semen has this characteristic, hierarchical structure in its ejaculate's organization. Regarding the GM-CSF actions, the existence of separate sensitivities to the effector depending on the specific subpopulation is noteworthy. Thus, the improving effects on Subpopulations 2 and 3 seem to be more marked than those on Subpopulations 1 and 4. This seems to indicate a separate response of the sperm in each subpopulation to the GM-CSF's effects. This subpopulation-dependent response could be linked to separate function rhythms of sperm included in each subpopulation. In fact, similar differences have been reported in other species, so, in

this way, dog sperm showed specific, separate responses against cell activators like glucose or fructose [37], thus indicating separate sensitivities to these activators. We do not know what the physiological implications that these function differences among ejaculates can have on sperm function are. Notwithstanding, they will be taken seriously when designing the utilization of factors like the GM-CSF in order to modulate and improve sperm viability in all conditions.

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