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Original Article

Effect of genistein supplementation of thawing medium on characteristics of frozen human spermatozoa

Juan Carlos Martinez-Soto[1, 2], Juan de DiosHourcade[3], Alfonso Gutiérrez-Adán[3], José Lorenzo Landeras[2], Joaquín Gadea[1]

[1]*Department of Physiology, School of Veterinary, Murcia University, Murcia 30100, Spain*
[2]*IVI- MURCIA, Murcia 30007, Spain*
[3]*Department of Animal Reproduction, INIA, Madrid 28040, Spain*

Abstract

In this study, we evaluated the effects of genistein supplementation of the thawing extender on frozen-thawed human semen parameters. We analyzed the effect of supplementation on sperm motility, capacitation (membrane lipid disorder), reactive oxygen species (ROS) generation, chromatin condensation and DNA damage. Using this preliminary information, it maybe possible to improve the cryopreservation process and reduce the cellular damage. We have confirmed that the isoflavone genistein (10 μmol L^{-1}) has antioxidant properties on the frozen-thawed spermatozoa. This results in a decreased ROS production that shows a slight improvement in the sperm motility, and decreases the membrane lipid disorder and DNA damage caused by cryopreservation. These results suggest an effect of genistein on sperm functionality that could be of interest for assisted reproduction treatments using frozen-thawed human spermatozoa, but further studies will be necessary to confirm our findings and to evaluate the possible clinical applications.

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1 Introduction

Sperm cryopreservation is performed in assisted reproduction centers, andrology laboratories and sperm banks. Cryopreservation of human semen represents a useful therapeutic option in the management of infertility with several indications [1]. However, during cryopreservation, spermatozoa are exposed to physical and chemical stress that results in adverse changes in membrane lipid composition, sperm motility, viability and acrosome status [2–6]. All these changes reduce the fertilizing ability of human spermatozoa after cryopreservation [7].

Cold shock during sperm cryopreservation is associated with oxidative stress and reactive oxygen species (ROS) generation [8]. ROS-induced damage to spermatozoa is mediated by oxidative attack of bis-allylic methylene groups of sperm phospholipid-bound polyunsaturated fatty acids (PUFAs), leading to lipid peroxidation [9, 10]. The effects of lipid peroxidation include irreversible loss of motility, leakage of intracellular enzymes, damage to sperm DNA [11] or deficiencies in oocyte penetration and sperm-oocyte fusion [12].

Genistein is an isoflavone found in soya and other legumes that has a direct effect on the function of mature spermatozoa by modifying the capacitation and acrosome reaction process. Genistein shows estrogen activity and inhibits protein tyrosine kinases [13]. Ty-

Correspondence to: Dr Joaquín Gadea, Department of Physiology, School of Veterinary, Murcia University, Murcia 30100, Spain.
Fax: +34-868-884-147 E-mail: jgadea@um.es
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rosine phosphorylation has been reported to have a key role in various aspects of sperm function, one of them being the process of sperm capacitation [14]. In addition, several studies have been carried out analyzing the antioxidant potential of isoflavones *in vitro* and *in vivo* [15, 16]. On the other hand, genistein has a protective antioxidant effect on sperm DNA integrity, assessed *in vitro* after hydrogen peroxide-mediated damage using the comet assay [17].

During cooling and freezing, the sperm membranes develop alterations in the structure and function, in some way similar to the capacitation process, which eventually reduce the life span of the sperm [18]. The possibility to modulate the capacitation process using exogenous molecules could increase the time of application of the cryopreserved spermatozoa with success. However, there is a lack of information about the capacity to modulate the capacitation and induce acrosome reaction, and the possible secondary damage of the use of these compounds on sperm functionality [19, 20].

The aim of this study was to evaluate the effect of supplementation of the dilution medium after thawing with genistein on frozen-thawed human spermatozoa. We evaluated the effect of such supplementation on sperm motility, capacitation (membrane lipid disorder), ROS generation, chromatin condensation and DNA damage. Using this preliminary information it maybe possible to improve the cryopreservation process and reduce the cellular damage.

2 Materials and methods

2.1 Sampling collection and preparation

This study was developed following institutional approval from the Instituto Valenciano de Infertilidad (IVI) and after obtaining an informed consent from men who visited our center for infertility screening.

Semen samples were obtained by masturbation and collected into sterile containers after 3–5 days of abstinence from sexual activity. After 10–15 min of liquefaction at 37ºC with 5% CO_2 in air, semen samples were examined for volume, sperm concentration, morphology and motility according to the World Health Organization (WHO) guidelines [21].

2.2 Cryopreservation of semen samples

Semen samples from 20 men who visited our center for infertility screening were frozen in pellets on the surface of dry ice using a glycerol-based cryoprotectant with egg yolk (Freezing Medium-Test yolk Buffer; Irvine Scientific, Santa Ana, CA, USA), as previously described [22, 23], with slight modifications. Briefly, seminal plasma was removed by centrifugation (10 min at 400 × *g*) and the spermatozoa were diluted (1:1 v/v) by slow addition of a glycerol-based cryoprotectant medium with continuous shaking. The diluted sperm samples were cooled up to 4ºC in a 90-min period and the sample was transferred on to a dry ice surface, in which small dimplets or dents (~50 μL) were engraved. After 2 min (when semen was totally frozen), all the pellets formed were transferred into a cryotube immersed in liquid nitrogen and then immediately stored in the semen banks for long-term storage.

The sperm freezing protocol has been employed since 1996, when the IVI clinic group reported the first full-term pregnancy achieved with frozen sperm obtained by testicular sperm extraction. To date, thousands of live births have been achieved in IVI facilities [24]. Cryopreservation in pellets offers some advantages over other packaging systems. These pellets have a higher surface-to-volume ratio with important implications for cooling, freezing and thawing rates of the semen [24, 25]. Pellet freezing of domestic animal semen generally produces the best results, but there are commercial pressures to use other methods of packaging [27].

2.3 Preparation of genistein solutions

Genistein (G-6649, Sigma-Aldrich Quimica, Madrid, Spain) was dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich Quimica) to yield a 1 mmol L^{-1} stock solution. This solution was frozen in aliquots and stored at –20ºC until use. The 0.1, 1 and 10 μmol L^{-1} working solution was prepared at the time of use with freezing or thawing medium. The final concentrations of DMSO in the samples treated with 0.1, 1 and 10 μmol L^{-1} genistein were 0.001%, 0.01% and 0.1%, respectively.

2.4 Thawing process

Aliquots of the samples were thawed in Human Tubal Fluid (HTF) + Human Serum Albumin ([HSA] 9:1; SAGE Coopersurgical, Trumbull, CA, USA) medium supplemented with or without genistein at 37ºC (final concentration 0, 1 and 10 μmol L^{-1} from stock solution of 1 mmol L^{-1} genistein in DMSO). Briefly, three pellets per sample (total 150 μL) were transferred to new tubes containing 300 μL of the thawing media (HTF + HSA supplemented or not with genistein) at 37ºC and maintained at this temperature for 60 min

before analysis. The samples were then washed and resuspended in phosphate-buffered saline (PBS) medium for analysis.

2.5 Motion parameters

Motion parameters were determined using a computer-assisted sperm analysis (CASA) system (ISAS, Valencia, Spain). The CASA-derived motility characteristics studied were percentage of motility and progressive motility, curvilinear velocity (VCL, $\mu m\ s^{-1}$), straight-line velocity (VSL, $\mu m\ s^{-1}$), average path velocity (VAP, $\mu m\ s^{-1}$), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), amplitude of lateral head displacement (ALH, μm) and beat cross-frequency (BCF, Hz).

A 7-μL drop aliquot of the sperm sample was placed on a pre-warmed (37ºC) slide (Superfrost Menzel-Glaser, Braunschweig, Germany) and covered with a 24 × 24 mm cover-slip. The setting parameters were: 25 frames of which the spermatozoa had to be present in at least 15 in order to be counted; the images were obtained at × 200 magnification in a phase contrast microscope. Spermatozoa with a VAP < 10 $\mu m\ s^{-1}$ were considered immotile. Minimum of five fields per sample were evaluated, counting a minimum of 200 spermatozoa per subsample.

2.6 Analysis of seminal parameters by flow cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, FL, USA). The fluorophores were excited by a 15-mW argon ion laser operating at 488 nm. Data from 10 000 events per sample were collected in list mode, and four measures per sample were recorded. Flow cytometric data were analyzed using the program Expo32ADC (Beckman Coulter Inc.) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

2.7 Assessment of membrane lipid packing disorder

To detect increase in plasma membrane lipid packing disorder, sperm samples were stained with merocyanine 540 (M540) and Yo-Pro1 [28]. Stock solution of M540 (1 mmol L^{-1}, Sigma-Aldrich Quimica) and Yo-Pro1 (25 μmol L^{-1}, Invitrogen) were prepared in DMSO. For each 1 mL diluted semen sample, 2.7 μL of M540 stock solution was added (final concentration of 2.7 μmol L^{-1}) and 1 μL of Yo-Pro (25 nmol L^{-1} final concentration). M540 fluorescence was collected with an FL2 sensor using a 575-nm band-pass filter and Yo-Pro1 fluorescence with an FL-1 sensor using a 525-nm band-pass filter. Cells were classified in three categories: low merocyanine fluorescence (viable, low disorder), high merocyanine fluorescence (viable, high disorder) or Yo-Pro1 positive (dead). Data are shown as the ratio of viable high lipid disorder/total viable (%) as an index of sperm capacitation.

2.8 Production of ROS

Production of ROS was measured by incubating the spermatozoa (1×10^6 sperms mL^{-1}) in PBS in the presence of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen) (0.5 μmol L^{-1}) for 60 min at 37ºC [30]. This dye is a fluorogenic probe commonly used to detect cellular ROS production. H_2DCFDA is a stable cell-permeable non-fluorescent probe. It is de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescein upon oxidation. Green fluorescence was collected with an FL1 sensor using a 525-nm band-pass filter. Measurements were expressed as the mean green intensity fluorescence units (mean channel in the FL1) and it was used as index of ROS generation.

2.9 Determination of chromatin condensation

Sperm chromatin was stained with propidium iodide (PI) for the determination of sperm chromatin condensation [30]. Thawed samples, after 60 min of incubation in the experimental media, were centrifuged (1 200 × *g*, 3 min) and the pellet was resuspended in a solution of ethanol and PBS (70/30, v/v) for sperm membrane permeabilization. The sample mixture was stored overnight at −20ºC until evaluation. After that, the samples were centrifuged, the supernatant discarded and the pellet was resuspended in a PI solution (10 mg mL^{-1} PBS). Samples were maintained in darkness for 1 h before flow cytometric analysis. Red PI fluorescence was collected with an FL3 sensor using a 650-nm band-pass filter. Measurements were expressed as the mean red intensity fluorescence units (mean channel in the FL3) and it was used as index of the state of the chromatin condensation, as this is inversely related to the PI uptake by DNA.

2.10 Evaluation of sperm DNA fragmentation by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL)

TUNEL staining was used to determine sperm

DNA fragmentation [31]. In brief, the cells were concentrated by centrifugation, fixed in a solution of ethanol and PBS (70/30, v/v) and stored overnight at −20ºC until evaluation. Cells (1×10^6 spermatozoa per mL) were washed twice with PBS and resuspended with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and labeled nucleotides (*In situ* cell death detection kit fluorescein; Roche diagnostics, Mannheim, Germany). The cells were incubated with the reaction buffer for 60 min at 37ºC, rinsed twice and measured by flow cytometry. Green fluorescence was collected with an FL1 sensor using a 525-nm band-pass filter and two populations were determined. The cells with fragmented DNA presented an intense green nuclear fluorescence. Cells were classified into two categories according to the intensity of fluorescence: low green fluorescence (low DNA damage) and high green fluorescence (high DNA damage). In addition, measurements were expressed as the mean green intensity fluorescence units (mean channel in the FL1) and it was used as an index of the DNA fragmentation.

2.11 Evaluation of sperm DNA fragmentation by neutral comet assay

Neutral comet assay was realized to evaluate double strand breaks in frozen-thawed sperm [32]. Spermatozoa were suspended in 1 mL of saline solution (10^6 spermatozoa per mL). A total of 10 µL of this mixture was mixed with 90 µL of low-melting agarose (1% w/v, Trevigen, Gaithsburg, MD, USA). Of this preparation, 75 µL was applied to the surface of a precoated (1% normal-melting agarose, dehydrated; LM-2, Hispanlab, Madrid, Spain) microscope slide and covered with a cover-slip (24 mm × 60 mm), allowing it to solidify for 20 min at 4ºC. Then the cover-slips were gently removed and an additional aliquot of low-melting agarose was placed on the slide. Slides were then submerged in cell lysis buffer (Trevigen) supplemented with dithiothreitol (40 mmol L^{-1}, Sigma-Aldrich Quimica) and Proteinase K (500 µg mL^{-1}, Sigma-Aldrich Quimica) for 150 min at 37ºC protected from light. Following cell lysis, all slides were washed through electrophoresis buffer (TBE, 50 mmol L^{-1}; TRIS, 50 mmol L^{-1}; sodium borate, 1 mmol L^{-1}; EDTA [pH 8], Sigma-Aldrich Quimica) for 5 min. Slides were placed in a horizontal electrophoresis unit and were allowed to equilibrate for 20 min with TBE buffer before running electrophoresis (25 V, 300 mA) for 12 min. When electrophoresis was complete, the slides were immersed in Tris-HCl (pH 7.4) for 5 min, rinsed with water, air-dried and stored protected from light until analysis.

Microgels were stained with DNA fluorochrome SYBR Green (1:10 000 dilution; Molecular Probes) and a cover-slip (24 mm × 60 mm) was applied before image analysis. Cells were visualized at × 200 using an epifluorescence microscope (Optiphot 2; Nikon, Tokyo, Japan). Quantitative image analysis was performed using a CCD camera (Nikon Coolpix S5000; Nikon) attached to the microscope. Comets were analyzed by CometScore free-software (TriTek Inc., Northern Virginia, Washington DC, USA). Tail length (µm), tail DNA (%), head DNA (%), tail moment and olive tail moment were recorded for 100 cells per sample [32]. The parameters that allowed us to describe extension of DNA damage were the tail moment and olive tail moment that were automatically obtained by the computer software system for each cell analyzed. Tail moment is calculated as the product of the tail length (µm) and the fraction of DNA in the tail (Tail %). Olive tail moment represents the product of the percentage of total DNA in the tail (Tail %) and the distance between the centre of the mass of head and tail regions (olive Tail Moment = [tail mean − head mean] × percentage of DNA in the tail).

2.12 Preliminary study

In a preliminary study, we evaluated the addition of genistein to the freezing media at different concentrations (0, 0.1, 1 and 10 µmol L^{-1}) on motion parameters evaluated by CASA. With the addition of low concentrations of genistein to the freezing media (0.1 and 1 µmol L^{-1}) no effect was detected on sperm characteristics. Only the highest concentration of genistein (10 µmol L^{-1}) showed a negative effect on sperm total and progressive motility (Table 1, $P < 0.01$) and on the velocities of the sperm parameters (VCL, VSL and VAP, Table 1, $P < 0.05$). In the next set of experiments, 1 and 10 µmol L^{-1} of genistein were added to the thawing media.

2.13 Experimental design

Frozen samples without genistein were thawed in the extender HTF + HAS supplemented with or without addition of 1 µmol L^{-1} or 10 µmol L^{-1} of genistein and maintained at 37ºC for 60 min. After this period, standard semen parameters and sperm functionality were evaluated, namely, (i) Motility and

motion parameters by CASA; (ii) Lipid membrane disorder status using M540 and viability using Yo-Pro1 by flow cytometry; (iii) Reactive oxygen formation by H_2DCFDA staining; (iv) Chromatin condensation by PI staining; (v) DNA fragmentation by TUNEL and (vi) by the neutral comet assay.

2.14 Statistical analysis

Data are expressed as the mean ± SEM. and analyzed by analysis of variance (ANOVA), considering the specific sperm treatment and men as the main variables. When ANOVA revealed a significant effect, values were compared by the least significant difference pair-wise multiple comparison Tukey *post hoc* test. Differences were considered statistically significant at $P < 0.05$. The analysis was conducted using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

3 Results

Addition of genistein (10 μmol L^{-1}) to the thawing media increased the percentage of motile and progressive motile spermatozoa (Table 2, $P = 0.01$). However, the spermatozoa treated with genistein (1 and 10 μmol L^{-1}) showed lower values for BCF than those in controls (Table 2, $P = 0.03$). No differences were found for the other motion parameters studied between control and experimental genistein groups. However, a tendency to reduce the values for VCL, VAP and STR was detected in the 1 μmol L^{-1} genistein group (Table 2, $P = 0.11$, 0.10 and 0.09, respectively).

In the same way, the addition of genistein (10 μmol L^{-1}) to the thawing media reduced the ROS generated during 60 min of incubation at 37ºC (Table 3, $P < 0.01$) and the percentage of high lipid disordered membrane with reference to viable spermatozoa, used as an index of sperm capacitation, was slightly reduced in both genistein groups (Table 3, $P < 0.01$).

The grade of chromatin condensation measured by the PI staining decreased in the 1 μmol L^{-1} genistein group compared with control (Table 4, $P = 0.02$), as this is inversely related to the PI uptake by DNA. When DNA damage was measured by TUNEL in both genistein groups, the percentage of sperm with DNA damage was lower than the control group (Table 4, $P < 0.01$) and this also reduced the mean fluorescents units in the10 μmol L^{-1} genistein group, which is an index of the number of DNA break points (Table 4, $P < 0.01$). Neutral comet assay confirmed the previous result and showed a lower DNA damage in the groups of genistein supplementation in all the parameters studied without difference between 1 and 10 μmol L^{-1} genistein groups, except for tail length (Table 5, $P < 0.01$).

4 Discussion

Sperm cryopreservation represents a common and important process in fertility treatments. During the process of freezing and thawing, physical and chemical stress to the sperm membrane are produced, leading to loss of sperm viability and fertilizing ability [2]. ROS are produced by a wide variety of exogenous chemicals and metabolic processes causing a broad spectrum of damage to the biological system. Sperm membrane lipid peroxidation has been shown to increase during cryopreservation, probably due to the release of oxygen radicals [33]. Spermatozoa are especially vulnerable to ROS-induced damage due to their PUFA content, and the use of antioxidants could reduce the negative ROS impact on spermatozoa [34, 35].

Several studies have been carried out examining the antioxidant potential of isoflavones, including genistein, on human spermatozoa and the induced DNA damage. *In vitro* effect of genistein is under controversy due in part to differences in experimental conditions. A first series of studies developed with samples from only one donor [36–38] showed a negative effect of genistein (1–250 μmol L^{-1}) on the DNA integrity of spermatozoa measured by comet assay in a similar response to H_2O_2, suggesting the hypothesis that genistein could generate ROS that target DNA [38]. However, other studies [15, 17] showed that use of genistein at lower concentrations (0.01, 0.1, 1, 10 and 100 μmol L^{-1}), produced a significant reduction in DNA damage after cells had been damaged by treatments with hydrogen peroxide. Recently, Bennetts *et al*. [39] reported that using fresh human spermatozoa that are incubated for only 15 min, in presence of higher concentrations of genistein (31 to 500 μmol L^{-1}) than those in our study, detected an increased ROS generation only with 500 μmol L^{-1} of genistein, but did not affect the viability or induce damage in the DNA measured by TUNEL. These authors suggested that estrogen analog compounds that did not possess vicinal hydroxyl groups, such as bisphenol A and genistein, were also fundamentally inactive in the induction of a high level of redox activity, although the latter generated a slight response at the highest dose tested [39].

Table 1. Motility parameters measured by CASA. Human frozen spermatozoa with freezing medium supplemented with GEN or not.

Freezing media	Percentage of progressive motility	Percentage of motility	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Control										
0 μmol L^{-1} GEN)	23.20 ± 1.27^{a}	25.59 ± 1.26^{a}	55.11 ± 1.46^{ab}	31.63 ± 1.06^{a}	37.86 ± 0.99^{a}	58.74 ± 1.49	81.85 ± 1.20	70.09 ± 1.12	1.10 ± 0.06	7.74 ± 0.29
0.1 μmol L^{-1} GEN	22.99 ± 1.42^{a}	25.98 ± 1.42^{a}	57.77 ± 1.85^{a}	31.86 ± 1.05^{a}	38.93 ± 1.04^{a}	58.12 ± 1.48	81.11 ± 1.21	69.93 ± 1.10	1.24 ± 0.08	7.82 ± 0.34
1 μmol L^{-1} GEN	20.44 ± 1.06^{ab}	22.98 ± 1.08^{ab}	53.20 ± 1.62^{ab}	29.97 ± 0.85^{ab}	36.72 ± 0.91^{ab}	59.21 ± 1.48	81.30 ± 130	71.14 ± 1.11	1.07 ± 0.07	7.65 ± 0.33
10 μmol L^{-1} GEN	17.22 ± 1.06^{b}	20.33 ± 1.21^{b}	51.44 ± 1.60^{b}	28.08 ± 0.93^{b}	33.94 ± 0.97^{b}	56.43 ± 1.48	81.84 ± 1.05	67.63 ± 1.13	1.11 ± 0.07	7.74 ± 0.35
Analysis of variance	*P*-values									
Freezing media	< 0.01	< 0.01	0.04	0.02	< 0.01	0.64	0.99	0.20	0.28	0.93
Men	< 0.01	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Interaction	< 0.01	< 0.01	0.03	0.09	< 0.01	0.68	0.71	0.22	0.07	0.09

Abbreviations: CASA, computer-assisted sperm analysis; GEN, genistein; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity of the curvilinear trajectory; STR, straightness; WOB, Wobble (VAP/VCL); ALH, amplitude of lateral head displacement; ; BCF, beat cross-frequency.
Numbers with different superscripts differ signficantly within the columns ($P < 0.05$).

Table 2. Motility parameters measured by CASA. Human frozen spermatozoa thawed in HTF medium supplemented with GEN or not .

Thawing media	Percentage of progressive motility	Percentage of motility	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Control										
0 μmol L^{-1} GEN	14.85 ± 0.65^{a}	27.45 ± 1.42^{a}	45.58 ± 1.29	23.26 ± 0.87	29.90 ± 0.89	50.61 ± 1.18	75.44 ± 1.10	65.25 ± 0.79	1.23 ± 0.06	7.96 ± 0.28^{a}
1 μmol L^{-1}GEN	14.99 ± 0.85^{ab}	28.07 ± 1.52^{ab}	42.22 ± 1.32	21.10 ± 0.93	27.76 ± 0.96	48.44 ± 1.37	72.19 ± 1.28	64.48 ± 1.01	1.14 ± 0.05	6.79 ± 0.29^{b}
10 μmol L^{-1}GEN	6.81 ± 0.87^{b}	29.57 ± 1.59^{b}	43.94 ± 1.30	23.31 ± 0.95	29.27 ± 0.94	51.36 ± 1.29	75.57 ± 1.17	65.93 ± 0.94	1.20 ± 0.06	7.05 ± 0.29^{b}
Analysis of variance	*P*-values									
Thawing media	0.01	0.01	0.11	0.83	0.10	0.24	0.09	0.54	0.54	0.03
Men	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Interaction	< 0.01	< 0.01	0.01	0.24	0.38	0.19	0.13	0.09	0.03	0.13

Abbreviations: CASA, computer-assisted sperm analysis; HTF, human tubal fluid; GEN, genistein; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity of the curvilinear trajectory; STR, straightness; WOB, Wobble (VAP/VCL); ALH, amplitude of lateral head displacement; BCF, beat cross-frequency.
Numbers with different superscripts differ significantly within the columns ($P < 0.05$).

Table 3. ROS generation (mean channel of fluorescence) and membrane lipid packing disorder. Human frozen spermatozoa thawed in HTF medium supplemented with GEN or not.

Thawing media	ROS	Ratio of high lipid disorder/total viable cells (%)
Control (0 μmol L^{-1} GEN)	19.11 ± 0.62^{a}	24.54 ± 1.93^{a}
1 μmol L^{-1} GEN	19.21 ± 0.56^{a}	23.59 ± 1.65^{b}
10 μmol L^{-1} GEN	18.40 ± 0.47^{b}	23.78 ± 1.93^{b}
Analysis of variance *P*-values		
Thawing media	< 0.01	< 0.01
Men	< 0.01	< 0.01
Interaction	< 0.01	< 0.01

Abbreviations: GEN, genistein; HTF, human tubal fluid; ROS, reactive oxygen species.
Numbers with different superscripts differ significantly within the columns ($P < 0.05$).

Table 4. Chromatin condensation measured by propidium iodide staining. DNA fragmentation by TUNEL (percentage of DNA damaged spermatozoa and mean green intensity fluorescence units). Human frozen spermatozoa thawed in HTF medium supplemented with GEN or not.

Thawing media	Chromatin condensation	TUNEL	
		percentage of damaged sperm	mean FU
Control (0 μmol L^{-1} GEN)	55.71 ± 4.90^{a}	5.09 ± 0.83^{a}	1.25 ± 0.34^{a}
1 μmol L^{-1} GEN	70.02 ± 6.07^{b}	4.45 ± 0.43^{b}	1.05 ± 0.26^{ab}
10 μmol L^{-1} GEN	62.41 ± 6.01^{ab}	4.63 ± 0.78^{b}	0.99 ± 0.22^{b}
Analysis of variance *P*-values			
Thawing media	0.02	< 0.01	< 0.01
Men	< 0.01	< 0.01	< 0.01
Interaction	< 0.01	< 0.01	< 0.01

Abbreviations: FU, fluorescent units; GEN, genistein; HTF, human tubal fluid; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling.
Numbers with different superscripts differ significantly within the columns ($P < 0.05$).

Table 5. Neutral comet assay. Human frozen spermatozoa thawed in HTF medium supplemented with GEN or not.

Thawing media	% DNA in head	% DNA in tail	Tail length	Tail moment	Olive tail moment
Control (0 μmol L^{-1} GEN)	67.26 ± 1.17^{a}	32.74 ± 1.17^{a}	35.69 ± 1.07^{a}	16.17 ± 0.99^{a}	10.91 ± 0.59^{a}
1 μmol L^{-1} GEN	70.88 ± 1.11^{b}	29.12 ± 1.11^{b}	28.67 ± 0.99^{b}	11.85 ± 0.80^{b}	8.27 ± 0.47^{b}
10 μmol L^{-1} GEN	73.04 ± 0.96^{b}	26.96 ± 0.96^{b}	32.82 ± 1.15^{a}	11.90 ± 0.78^{b}	8.31 ± 0.46^{b}
Analysis of variance *P*-values					
Thawing media	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Men	0.02	0.02	< 0.01	< 0.01	< 0.01
Interaction	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Abbreviations: GEN, genistein; HTF, human tubal fluid.
Numbers with different superscripts differ significantly within the columns ($P < 0.05$).

Under our experimental conditions, using frozen-thawed spermatozoa, the addition of genistein to the thawing media moderated the ROS generated and had a protective effect on sperm DNA, showing a lower DNA damage in the groups of genistein supplementation. These data suggest that genistein used at these concentrations (1 μmol L^{-1} and 10 μmol L^{-1}) could offer antioxidant properties to the frozen-thawed spermatozoa. The lower DNA damage observed following supplementation of the thawing medium with genistein suggests that ROS generation and redox balance are the most important factors responsible for the disruption of the condensation and stability of sperm chromatin after cryopreservation [40]. Recently, Thomson *et al*. [41] reported that addition of 50 μmol L^{-1} and 100 μmol L^{-1} of genistein to the cryoprotectant had a significant protective effect on sperm DNA using TUNEL and 8OHdG as a biomarker of oxidative stress and they also reported a significant

increase in motility and viability. These authors concluded that human sperm DNA fragmentation is associated with an increase in oxidative stress during cryopreservation, rather than the activation of caspases and apoptosis [41].

Previous studies in human and domestic animal spermatozoa have shown that freezing and thawing induce important changes in the sperm chromatin, resulting in greater compactness [30, 31, 42, 43]. Thus, chromatin condensation and stability may be critical factors to consider when using frozen semen [44]. In this study, we observed a lower condensation of the nucleus of the spermatozoa treated by 1 μmol L^{-1} of genistein. This fact would be related to a lower condensation in the thawing process due to the antioxidant effect of genistein against the high level of ROS generated during the thawing process. Concerning this mechanism, interaction of sperm chromatin with ROS such as H_2O_2 could lead to sulphydryl group oxidation [45] and to an increase in disulfide bond cross-linking and higher chromatin compactness. Genistein would block the putative effect of H_2O_2, leading to a lower degree of compactness and DNA damage. Nevertheless, further studies will be necessary to confirm these preliminary findings and determine the best concentration of genistein for protecting DNA in a higher number of patients.

The initiation of the sperm capacitation process is related to an alteration in the redox balance between ROS generation and the activity of the antioxidant defence mechanisms [9, 35]. On the other hand, sperm protein tyrosine phosphorylation has been associated with capacitation, motility changes and hyperactivation. The inhibition of tyrosine kinase produces a decrease in the magnitude of motion parameters [19]. It is well known that genistein is a tyrosine kinase inhibitor with an effect on sperm motility in a dose-dependent manner. Hence, Kumi-Diaka *et al*. [46] reported that low doses of genistein did not influence mice sperm motility, but high concentrations of genistein interfered with percentage sperm motility, and it was confirmed in human spermatozoa [19, 20], thereby showing that incubation of sperm with 400 μmol L^{-1} genistein decreases the sperm motility. In our study, we detected slight increase in motility (progressive and total) when genistein (10 μmol L^{-1}) was present in the thawing media; however, there was a tendency to modify the pattern of movement (VCL, VAP, STR, BCF) when 1 μmol L^{-1} of genistein was added. This fact could be related to the inhibition of tyrosine kinase and capacitation process [19]. In the future, it is necessary to evaluate the different concentrations of genistein (in the range between 1 μmol L^{-1} and 10 μmol L^{-1}) supplemented in the freezing and thawing medium, because it is important to know the concentration in which the decreased motility parameters are observed.

Opposing results are observed when genistein (10 μmol L^{-1}) is added to freezing or thawing media. It could be related to the pro-oxidative and anti-oxidative capacity that the antioxidant substances present at a different concentration, thereby producing paradoxical effects. In our previous experiments with respect to antioxidant addition of reduced glutathione (GSH) to freezing and thawing media [29–31, 48], we detected more significant activities when the antioxidant was added in the thawing, than in the freezing media. A plausible explanation for this question would be that cryodamage of antioxidant enzymes during the freezing process results in an overall decrease of enzymatic antioxidant defences in the sperm. This damage could selectively affect superoxide dismutase (SOD), glutathione reductase (GRD) and glutathione peroxidase to a lesser extent. During thawing, there is an increase in the production of ROS, with an increase in the superoxide anion production due to a decrease in SOD activity and a decrease in GSH content due to a decrease in GRD activity and an increase in GSH oxidation by hydrogen peroxide. A decrease in intracellular GSH and an increase in ROS production during thawing would lead to an increase in lipid peroxidation. Genistein provided exogenously after thawing would prevent hydrogen and lipid peroxide-induced damage and maintain the motility function.

Membrane lipid disorder is one of the early signals of the process of sperm capacitation [28]. In our experimental conditions, we detected a slight reduction in the percentage of viable sperm with high lipid membrane disorder. The role of genistein in capacitation is not well defined; Fraser *et al*. [49] reported that genistein at low concentrations (1, 10 and 100 nmol L^{-1}) accelerated the capacitation and acrosome loss in human spermatozoa measured by CTC staining. On the other hand, several studies have shown the capacity of genistein taken at a higher concentration to inhibit the progesterone-induced acrosome reaction [50–52]. Besides these differences related to concentration, another question to take into consideration is related to the possible different mechanisms for induced

capacitation of fresh spermatozoa and cryocapacitation, as previously described in bull spermatozoa [53]. In the frozen bull spermatozoa, the genistein did not affect tyrosine phosphorylation [54], suggesting that cryopreservation affects the regulatory mechanism of capacitation [53, 54].

The use of techniques such as flow cytometry and CASA allowed to evaluate sperm functionality in a specific, objective, accurate and reproducible method compared with traditional microscopy-based methods [55, 56] and to detect slight significant differences. On the other hand, using two-way ANOVA analysis, we are able to evaluate the effect of the treatment and distinguish it from the potential effect of an individual man. For most of the seminal parameters, we noticed that men and interaction are statistically significant ($P < 0.05$, Tables 1–5). To elucidate whether the differences found between treatments could be driven by few samples or whether it was a general phenomenon, we analyzed the proportion of samples that increased or decreased the values and found that most of the samples (range 65%–85%, 13 to 17 in 20 samples) followed the same pattern (increasing or reducing the values) after treatment for every seminal parameters studied. However, there were differences in the intensity of these increases. In addition, it was analyzed whether the samples that presented a different pattern were associated with certain level of motility or other sperm parameters, but we did not find any relationship or association with it.

For a clinical application of treatment with isoflavones, it will be necessary to evaluate a higher number of samples and determine the possibility that some groups of patients could show a more pronounced effect. However, this topic is out of the aim of this paper and it must be studied deeply in the future.

In summary, in this study we have confirmed that the isoflavone, genistein, in the range of 1–10 μmol L^{-1} concentration has antioxidant properties in the frozen-thawed spermatozoa. These effects produce a decrease in ROS production that originates an improvement in the sperm motility and decreases DNA damage caused by the process of cryopreservation. These results suggest an effect of the genistein on the sperm functionality that could be of interest for assisted reproduction treatments with frozen-thawed human spermatozoa, but further studies will be necessary to confirm our findings and to evaluate the possible clinical applications.

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