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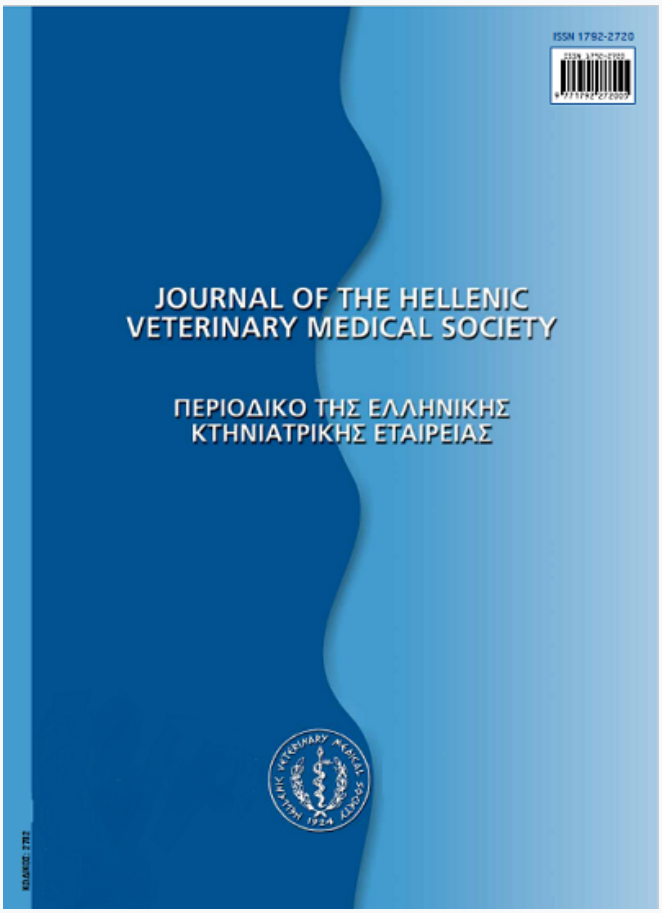

Effect of genistein addition to equine sperm freezing extender

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Research article
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Effect of genistein addition to equine sperm freezing extender

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ABSTRACT. Cryopreservation negatively affects equine sperm quality post-thaw: an excessive release of reactive oxygen species (ROS) and increased protein tyrosine phosphorylation (known as cryocapacitation) have been demonstrated in frozen-thawed equine sperm diminishing its lifespan. The objective of this study was to determine the possible beneficial effect of genistein addition (a ROS scavenger and protein tyrosine kinase inhibitor) to a commercial freezing extender. Equine sperm were frozen in the presence of different genistein concentrations ranging from 0 to 800 μM. After thawing, the sperm viability (eosin-nigrosin), acrosome integrity using peanut agglutinin (PNA), protein tyrosine phosphorylation (PY) and motility were assessed at times 0 and 1 hr. In addition PY was studied in fresh and frozen sperm incubated in Modified Whitten's (MW) medium with 25 mM Bicarbonate (MW+Bic). Genistein did not affect the viability, total or progressive motility or acrosome status of frozen-thawed equine sperm. Immediately after thawing, positive PY staining in the equatorial band was observed and genistein addition did not exert any change in PY. Fresh sperm incubated in MW+Bic showed a significant increase in PY staining along the tail compared to frozen-thawed sperm. In our study sperm viability immediately post-thaw was 58.25% ± 5.35 and progressive motility 14.46% ± 5.7 (mean ± S.E.M.) and genistein did not improve the post-thaw quality of equine sperm. In addition, cryopreservation did not induce PY immediately post-thaw or enhanced frozen-thawed equine sperm susceptibility to PY induction.

Keywords: antioxidants, cryopreservation, sperm, equine

INTRODUCTION

Cryopreservation induces deleterious effects in equine sperm due to the concurrence of several phenomena during freezing-thawing cycles namely ice crystal formation, osmotic shock, cold shock and an increased production of reactive oxygen species (ROS) (Hammerstedt et al., 1990; Pommer et al., 2002; Sieme et al., 2008). These events diminish sperm viability, causing a notable decrease of sperm quality compared to fresh semen, resulting in impaired fertility (Metcalf, 2007). The use of frozen-thawed stallion semen involves additional costs for the owners of the stallion and the mare; the lower the motility and viability of sperm, the higher the number of straws required per artificial insemination dose and also, more ultrasounds are needed to determine the exact timing of ovulation (Metcalf, 2007). One of the most important harmful changes occurring during freezing and thawing cycles in equine sperm is the increase in reactive

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oxygen species (ROS) production (Ball et al., 2001; Burnaugh et al., 2010). Low concentration of ROS are produced physiologically in mammalian sperm during capacitation, hyperactivation and acrosome reaction (de Lamirande et al., 1997). However, an excessive increase in ROS production results in DNA fragmentation (Aitken and Baker, 2006) and oxidative degradation of the highly unsaturated plasmalemma's lipids (Garcia et al., 2011), resulting in a decrease of the sperm's membrane integrity and functionality (Ball et al., 2001; Ball and Vo, 2002).

In frozen-thawed equine sperm, an increased ROS production, has been reported to induce severe changes such as DNA fragmentation (Baumber et al., 2003a). Among all the ROS produced by equine sperm, hydrogen peroxide has been claimed to be the most harmful being involved in enhanced acrosome reaction and induction PY induction in fresh equine sperm (Baumber et al., 2003b) and has also been shown to affect detrimentally the motility of fresh equine sperm (Baumber et al., 2000). It has also been demonstrated that ROS addition was associated with a marked increase of protein tyrosine phosphorylation (PY) in fresh equine semen (Baumber et al., 2003b). PY has been related to sperm fertilization ability and, more specifically, is known to be a final hallmark of mammalian sperm capacitation (Visconti et al., 1995). During sperm cryopreservation this PY increase can also be observed and is known as "cryocapacitation" (Cormier and Bailey, 2003), but far from being a physiological event that prepares sperm for fertilization, it leads to a decreased life span of mammalian sperm in the female reproductive tract (Bailey et al., 2000). The cryocapacitation process has been reported in the equine species (De Andrade et al., 2012; Schembri et al., 2002; Thomas et al., 2006). Several studies have shown that antioxidants neutralize the increase in ROS, and have been added to cryopreservation extenders in an attempt to avoid sperm damage resulting in excessive ROS production. However, compounds with antioxidant properties such us ascorbic acid and alpha-tocopherol (Baumber et al., 2005), or quercetin (Gibb et al., 2013) have been shown not to exert any benefit on equine sperm quality after thawing. In addition, other ROS scavengers such as glutathione have resulted in moderate beneficial effects in equine sperm (de Oliveira et al., 2013). Genistein is a phytoestrogen belonging to the isoflavone category with antioxidant properties and it is an effective hydrogen peroxide scavenger (Record et al., 1995) as well as a potent inhibitor of protein tyrosine kinases (Gonzalez-Fernandez et al., 2013). Genistein addition to cryopreservation media has shown to induce an increase in sperm motility and viability and a decrease in DNA damage in human sperm (Thomson et al., 2009). Therefore, the aim of this work was to improve equine sperm post-thaw quality. To fulfil this objective, different concentrations of genistein were added to a commercial freezing extender and the impact of genistein on viability, motility, PY and acrosome status post-thaw was assessed.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), Genistein, Triton X-100, poly-L-lysine and PNA-FITC conjugate were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Anti-phosphotyrosine monoclonal antibody (clone 4G10) was from Millipore (Billerica, MA, USA). Anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated secondary antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Slowfade® gold antifade was from Molecular Probes (Eugene, OR, USA).

Semen collection and processing

Semen was collected from five fertile pure bred Lusitano stallions, with ages ranging from 3 to 23 years, using an artificial vagina (Hannover model, Minitüb, Landshut, Germany). All experimental procedures were performed according to institutional and European regulations. A nylon in-line filter (Animal Reproduction Systems, Chino, CA, USA) was used to eliminate the gel fraction. The sperm-rich fraction was diluted 1:2 (v:v) in Kenney's medium and subsequently processed for cryopreservation.

Sperm cryopreservation and thawing

The ejaculates were centrifuged 10 min at 900 × g and the supernatant was discarded. Cryopreservation of equine sperm cells was performed as described by Guimarães et al. (2012). Briefly, the sperm pellet was re-suspended in an egg-yolk based freezing extender (Botucrio®, Botupharma, Botucatu, Brazil) added with the different genistein concentrations used (0, 10 μM, 100 μM, 200 μM, 400 μM and 800 μM) yielding a final concentration of 200 x 10^6 sperm/m*l*. Three straws (0.5 m*l*) per treatment were loaded and maintained at 4 ºC for 20 min. Then, the straws were placed 6 cm above the liquid nitrogen (LN_2) surface and kept in nitrogen vapours for further 25 min. After this period the straws were plunged in LN_2 and stored. Straws were thawed in a water bath, at 37ºC for 1 min, their content transferred into a pre-heated eppendorf tube at 37ºC and analyzed after 10 min (time 0) or after 1 hr (time 1 hr). Each was analysed individually.

Viability

For viability evaluation, the supravital eosin-nigrosin stain was used. Twenty microliters of semen were mixed with 20 μl of the stain, and the mix was smeared on a pre-heated slide at 37ºC. The samples were air dried and examined using a light microscope (magnification 100×). One hundred sperm were counted per sample. Sperm excluding the nigrosin-eosin stain were considered alive, while sperm showing a "pinkish" colour were counted as dead.

Motility analysis

Sperm motility was analyzed by a computer-assisted sperm analysis (CASAS) system (ISAS 1.0.6; Proiser S.L., Valencia, Spain). Sperm samples were diluted to 50 x 10^6 sperm/m*l* using Kenney's medium and an aliquot (6 μl) of each sample was placed in a pre-heated (37°C) motility chamber with a fixed height of 20 μm (Proiser S.L., Valencia, Spain). For motility assessment, a minimum of three microscopic fields and 300 total sperm were evaluated at 25 frames per second. The parameters assessed were percent of total motility (TM), percent of progressive motility (PM), curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straightness (STR), amplitude of lateral head movement (ALH), and linearity (LIN).

Protein tyrosine phosphorylation

Fresh or frozen-thawed sperm were centrifuged 5 min at 500 × g and washed in 1 m*l* of PBS. After centrifugation the pellet was resuspended in 1 m*l* of PBS and the cell concentration was adjusted to 5 × 10^6 spermatozoa/m*l*. Ten microliters of each sample was placed on a slide pre-treated with poly-L-lysine. After 10 min,

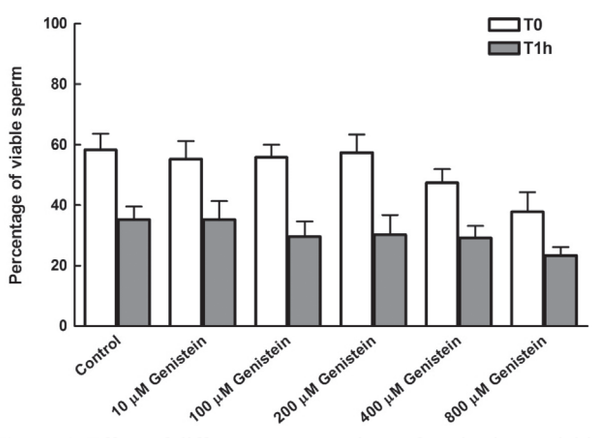

Figure 1. Effect of different concentrations of genistein on viability of frozen-thawed equine sperm. After thawing, sperm were incubated for 1 h at 37 ºC in air in freezing extender. No significant differences among incubation time (0 or 1 hour) were found ($p > 0.05$) (n = 5).

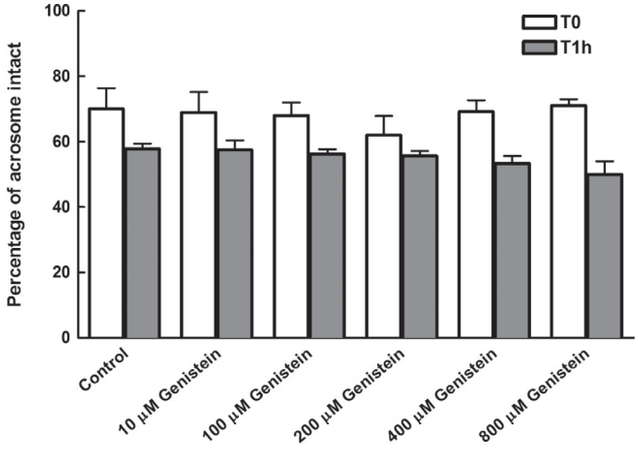

Figure 2. Effect of different concentrations of genistein on the acrosome status of cryopreserved equine sperm. After thawing sperm were incubated for 1 h at 37 ºC in air in cryopreservation extender. No significant differences among incubation time (0 and 1 h) or concentration used were found ($P > 0.05$) (n = 5).

Table 1. Effect of different concentrations of genistein on total (TM) and progressive motility (PM) in frozen-thawed equine sperm.

	Time 0		Time 1h	
	TM (% ± S.E.M.)	PM (% ± S.E.M.)	TM (% ± S.E.M.)	PM (% ± S.E.M.)
Control	23.7 ± 7.7[a]	14.4 ± 5.7[a]	13.1 ± 3.0[a]	5.9 ± 2.1[a]
10 µM genistein	23.1 ± 7.7[a]	12.6 ± 5.4[a]	11.7 ± 4.1[a]	5.4 ± 2.5[a]
100 µM genistein	17.1 ± 3.1[a]	8.8 ± 2.6[a]	10.7 ± 2.2[a]	4.8 ± 1.6[a]
200 µM genistein	16.5 ± 5.7[a]	9.0 ± 3.5[a]	9.5 ± 3.0[a]	4.3 ± 1.7[a]
400 µM genistein	17.6 ± 4.0[a]	8.5 ± 2.9[a]	8.0 ± 2.0[a]	3.4 ± 1.5[a]
800 µM genistein	12.5 ± 3.4[a]	5.7 ± 2.1[a]	5.2 ± 1.7[a]	1.6 ± 0.9[a]

After thawing, sperm were immediately analyzed (time 0) or incubated for 1 hr at 37 ºC in air in freezing extender. No significant differences among incubation times were found ($p > 0.05$) ($n = 5$).

spermatozoa were fixed with 4% formaldehyde in PBS for 15 min at room temperature and sperm were permeabilized with 0.1% Triton X-100 (v:v) in PBS for 10 min. After three washes with PBS, sperm were blocked with 3% BSA in PBS for 60 min. Primary incubation with antiphosphotyrosine monoclonal antibody (diluted 1:500) was performed in 3% BSA in PBS at 4°C overnight. The sample was then washed with PBS and incubated with anti-mouse IgG (FITC)-conjugated secondary antibody in 3% BSA in PBS for 1 hr at RT. After three washings with PBS, the samples were mounted on a slide with Slowfade® gold antifade solution from Molecular Probes following manufacturer's indications (Eugene, OR, USA). Samples were evaluated using a CyScope Plus HP-Partec fluorescence microscope equipped with 100× objective. One hundred sperm cells were evaluated and the number of stained tails counted.

Acrosome evaluation

Sperm were washed, fixed as described in the previous section and, after permeabilization for 10 min in PBS with 3% BSA and 0.1% Triton X-100, (v:v), were incubated with PNA-FITC (1 μg/m*l*) in PBS for 30 min at room temperature. The sample was then washed with PBS and mounted on a slide with Slowfade® gold antifade solution. Samples were evaluated using a CyScope Plus HP-Partec fluorescence microscope equipped with 100× objective. One hundred sperm per sample were examined, and the number of stained acrosome counted. Stained acrosomes were considered intact, as previously described (Silva and Gadella, 2006).

Statistical analysis

A one-way ANOVA was used to compare values, followed by a Tukey post-hoc test if differences were found. Statistical significance was set at $p < 0.05$. Analyses were performed using GraphPad Prism ver. 4 for Windows (GraphPad Software Inc., San Diego, CA, 2003).

RESULTS

Effect of genistein on sperm viability and motility parameters

Viability post-thaw in the control group was 58.25% ± 5.3 (mean ± S.E.M.), and total and progressive motility were 23.7% ± 7.7 and 14.4% ± 15.7 (mean ± S.E.M.) respectively. Our results showed that sperm viability as well as total and progressive motility were not affected by any of the genistein doses tested (Fig. 1 and Table 1; $p > 0.05$) or by the incubation length (time 0 and 1 hr; $p > 0.05$) when treatments were com-

Table 2. Effect of different concentrations of genistein on protein tyrosine phosphorylation in frozen-thawed equine sperm.

	Equatorial band (% ± S.E.M.)	
Treatment	Time 0	Time 1h
Control	9.2 ± 5.5[a]	3.8 ± 1.9[a]
10 μM genistein	7.8 ± 4.2[a]	3.6 ± 1.4[a]
100 μM genistein	7.4 ± 2.9[a]	5.4 ± 1.5[a]
200 μM genistein	7.2 ± 4.3[a]	2.8 ± 1.3[a]
400 μM genistein	8.8 ± 3.8[a]	4.6 ± 1.5[a]
800 μM genistein	11.6 ± 4.3[a]	4.2 ± 1.4[a]

After thawing, sperm were analyzed (time 0) or incubated for 1 hr at 37ºC in air in cryopreservation extender. No significant differences among incubation time were found (p > 0.05) (n = 5).

pared between their respective incubation times.

Effect of genistein on acrosome status

After thawing, equine sperm showed 70.0% ± 6.4 (mean ± S.E.M.) of acrosome intact sperm. Our results showed that the addition of genistein at the different concentrations used (10 - 800 μM), did not significantly change the percentage of sperm showing an intact acrosome ($p > 0.05$; Fig. 2) after freezing and thawing at time 0 or after 1 hr incubation in freezing extender.

Effect of genistein on protein tyrosine phosphorylation:

Immunolocalization of PY in frozen-thawed equine sperm

In situ immunofluorescence was performed to localize tyrosine-phosphorylated proteins in equine sperm after thawing (time 0) or after 1 hr incubation in cryopreservation extender at 37°C in air. Frozen-thawed sperm incubated in cryopreservation extender showed a solely fluorescence pattern consisting in a positively stained equatorial band (Fig. 3, A-B) and did not show differences immediately (time 0) or after 1 hr incubation in freezing extender (Table 2).

PY induction in frozen-thawed equine sperm

In view of the previous results, we wanted to elucidate if an increase in PY can be induced in frozen-thawed equine sperm and if genistein addition affects this phenomenon. To achieve this goal, we incubated fresh or thawed equine sperm in MW+Bic at 10 x 10^6 sperm/m*l*, a medium that has consistently been shown to induce protein tyrosine phosphorylation in fresh equine sperm (Gonzalez-Fernandez et al., 2012a). Fresh and frozen-thawed equine sperm incubated for 1 hr in MW+Bic showed an additional phosphorylation pattern along the entire tail (Fig. 3, C-D) or entire tail with equatorial band (image not shown). For analysis, sperm showing positive stained tail or equatorial band with stained tail were combined and considered as positive PY tail staining. The highest value for PY immunostaining was found in fresh sperm (28.5% ± 2.6; mean ± S.E.M.) and this was significantly different from the rest of treatments ($p < 0.001$). Although there was a dose dependent-concentration decrease of PY in presence of genistein in frozen-thawed sperm, there were no significant differences between treatments except for the genistein dose of 800 μM compared to 10 μM (2.3% ± 0.6; mean ± S.E.M.) ($p < 0.01$; Table 3).

DISCUSSION

The present work was aimed to diminish the impact of sperm cryopreservation by the addition of genistein to a commercial freezing extender. In the first part of our study, viability and motility were assessed post-thaw. The control group showed 58.2% ± 5.3 viability and

Table 3. Effect of different concentrations of genistein on protein tyrosine phosphorylation in equine sperm.

Treatment	Tail stained (% ± S.E.M.)	Equatorial band (% ± S.E.M.)
Control (fresh)	28.5 ± 2.6^{a}	5.7 ± 1.0^{a}
Control (thawed)	10.7 ± 1.4^{bc}	2.7 ± 1.8^{a}
10 μM genistein	15.0 ± 1.5^{c}	2.7 ± 0.7^{a}
100 μM genistein	7.3 ± 1.5^{bc}	3.7 ± 2.3^{a}
200 μM genistein	9 ± 1.5^{bc}	4.0 ± 2.1^{a}
400 μM genistein	6.6 ± 1.2^{bc}	3.7 ± 0.7^{a}
800 μM genistein	2.3 ± 0.6^{b}	2.7 ± 0.7^{a}

Fresh (n = 4) or thawed sperm (n = 3) were incubated for 1 hr at 37ºC in air in MW+Bic at 10 x 10^{6} sperm/ml. PY waslocalized in the tail or in the equatorial band. Values bearing different superscripts in the same column, differ statistically (p < 0.01).

14.47% ± 15.7 progressive motility (mean ± S.E.M.). Our results showed that the addition of genistein to the freezing extender did not adversely influence the viability of equine sperm (Fig. 1) despite the broad range of dosages used (in agreement with González-Fernández et al. 2013), so we tried in our experiments to test its effect on motility, acrosome integrity and cryocapacitation in view of its low toxicity.

In our study, none of the genistein doses tested had any effect in frozen-thawed equine sperm motility (total or progressive; Table 1) or acrosome status (Fig. 2) either immediately or one hr after thawing ($p > 0.05$). These results are in contrast with those of Thomson et al. (2009), who found that addition of genistein to the freezing media (100 μM) significantly increased total motility of human sperm, while this effect was not appreciated in bull sperm, not even after 1 hr incubation (Menzel et al., 2007). Taking in account the above data we can suggest that the effect of genistein on sperm motility differs depending on the species used. In our experiments, even when high doses of genistein (up to 800 μM) were added to the freezing extender, motility parameters were not improved (Table 1). In addition, viability and acrosome status resulted unaffected regardless the dose of genistein used immediately or after 1 hr incubation (Fig. 1 and Fig. 2). These results are in agreement with those of Morillo-Rodriguez et al. (2012) and Baumber et al. (2005), who did not find any differences in viability, motility parameters or acrosome status after the addition of butylated hydroxytoluene, ascorbic acid or alpha-tocopherol to the freezing extender of equine sperm. Genistein is a very well-known general inhibitor of protein tyrosine kinases in equine sperm (Gonzalez-Fernandez et al., 2013), so its use in freezing extenders could theoretically help to modulate the PY that takes place after thawing (De Andrade et al., 2012). However, in our study, we did not observe an increase in PY after sperm thawing (time 0) or after 1 hr incubation in cryopreservation extender, so genistein did not induce any changes in PY (Table 2).

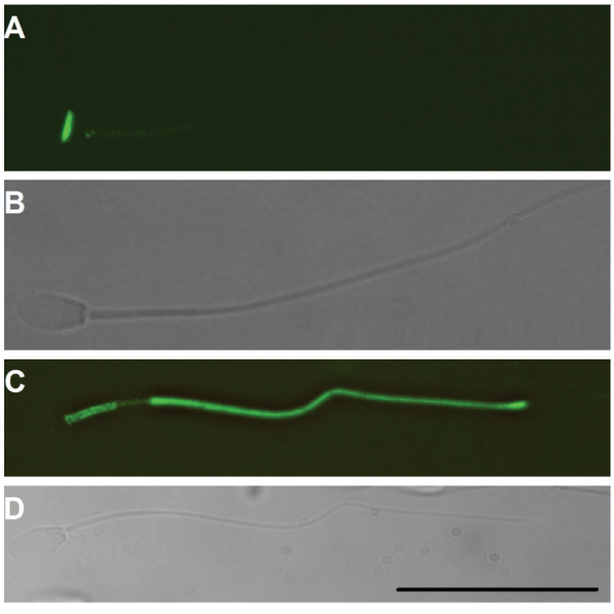

Figure 3. Immunofluorescence pattern of PY in frozen-thawed equine sperm. Sperm were incubated in freezing extender for 1 h at 37 ºC (A: fluorescence; B: bright field) or in MW+Bic for 1 h (C: fluorescence; D: bright field). Bar = 20 μm.

Pommer et al. (2003) postulated that thawed equine spermatozoa are more susceptible to undergo PY than fresh sperm when incubated under capacitating conditions. These results are not in agreement with those of Parker et al. (2000), who showed lower capacitation inductility *in vivo* of frozen-thawed equine sperm compared to fresh ejaculates measured by chlortetracycline staining. In our study, equine sperm were incubated in MW+Bic and no significant differences were found between the treatments concerning the frozen-thawed sperm ($p > 0.05$; Table 3). However, significant differences in PY positive staining were found between fresh (28.5% ± 2.6; mean ± S.E.M.) and thawed equine sperm (10.7% ± 1.4; mean ± S.E.M.) after 1 hr incubation in MW+Bic in contrast to the work by Pommer et al. (2003), while our results were in agreement with those of Parker et al. (2000).

In this regard, Thomas et al. (2006) found a different PY pattern using western blotting in fresh compared to frozen-thawed equine sperm, while De Andrade et al. (2012) reported a higher PY increase in frozen vs. fresh sperm using flow cytometry. Taken together, all the previously mentioned works and our results suggest that the increase in capacitation-like phenomena in frozen-thawed equine sperm likely varies depending on the methodological approach used. In addition, stallion to stallion variability to PY induction *in vitro* (Gonzalez-Fernandez et al., 2012a) and to withstand the freezing-thawing cycles (Loomis and Graham, 2008) can also explain this apparent divergence. Furthermore, as different breeds were used between studies, there might be differences among their cryocapacitation status or susceptibility, and these facts need to be also taken in to account. Nevertheless, our results did not show any significant increase in PY compared to fresh sperm, reflecting that cryopreservation does not increase PY in equine sperm nor enhances their susceptibility to PY induction. The fact that the addition of genistein does not significantly improve any of the parameters measured in the present work may suggest that excessive ROS production prior, or during freezing may not be the main change which put in risk the equine sperm quality. Instead, other damaging events like cold shock (Martorana et al., 2014) or structural damage due to osmotic shock (Gonzalez-Fernandez et al., 2012b) seem to be more important.

CONCLUDING REMARKS

In conclusion we demonstrated that in frozen-thawed equine sperm the use of genistein does not render any advantages in terms of viability, total or progressive motility, acrosome-intactness or modulation of PY. Therefore, to improve post-thaw sperm quality more effort has to be made to diminish the negative impact of other freezing-related changes.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.

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