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The inhibition of spermatic cystine/glutamate antiporter xCT (SLC7A11) influences the ability of cryopreserved stallion sperm to bind to heterologous zonae pellucidae

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Abstract

Sperm are redox-regulated cells, and deregulation of their redox status is considered to affect male fertility and to reduce their fertilizing ability following biotechnological procedures, such as cryopreservation. Cystine (CysS), after incorporation in sperm via SLC7A11 antiporter, has been demonstrated to increase intracellular GSH content, the most important non enzymatic antioxidant. This study was aimed at investigating the role of SLC7A11 antiporter on frozen-thawed stallion sperm ability to respond to in vitro capacitating environment after post-thaw incubation with CysS and/or Sulfasalazine (SS), a specific inhibitor of SLC7A11 antiporter. Viability, motility, immunolocalization of tyrosine phosphorylated proteins and the ability to bind to heterologous zonae pellucidae were evaluated. Thawed sperm from seven stallions (2 ejaculates/stallion) was washed and resuspended in Tyrodes media; each thawed ejaculate was divided in Control (CTR) and 3 samples supplemented with: 0.5 mM Cystine (CysS), 500 µM Sulfasalazine (SS) and 0.5 mM CysS + 500 µM SS (CysS+SS). After 1 h of incubation at 37°C, samples were washed twice, resuspended in capacitating BWW medium and incubated at 38 °C under 5% CO_2. After 30 and 60 min, sperm motility, viability and tyrosine phosphorylated protein immunolocalization, used as capacitation status index, were evaluated. After 30 min of capacitation, $4x10^5$ sperm were co-incubated with denuded pig oocytes in capacitation medium for 30 min for the heterologous binding assay. None of the sperm parameters studied (motility, viability and tyrosine phosphorylation) showed any difference respective to control. The number of sperm bound per oocyte (mean ± SEM) tended to increase in CysS group (44.0 ± 12.3) respect CTR (40.8 ± 10.8) while decreased in SS group (32.4 ± 7.8) ($p<0.01$). Moreover, CysS+SS group showed a lower binding rate (32.0 ± 10.0) compared to CysS ($p<0.001$). Our results suggest that CysS supplementation of thawed stallion sperm can influence their ability to bind to heterologous zona pellucidae as the inhibition of CysS incorporation by SLC7A11 reduced the number of sperm bound per oocyte. This effect does not seem to be ascribed to a modification of sperm motility, membrane integrity and tyrosine phosphorylation.

Keywords: Equine, Frozen semen, Zona pellucidae, Reactive oxygen species, Cystine

1. Introduction

Sperm are redox regulated cells. Controlled and physiological reactive oxygen species (ROS) amounts are required as signalling molecules for sperm crucial functions such as capacitation, acrosome reaction and sperm-oocyte interactions. However high ROS levels are considered the main cause of cellular damage induced by many sperm biotechnologies [1-5].

Most of the ROS formation takes place at the mitochondrial transport chain level during the process of oxidative phosphorylation (OXPHOS). Stallion sperm produce high amounts of ROS as they are highly dependent on OXPHOS for ATP production and are characterized by an unusually intense mitochondrial activity in comparison with those of other mammals [6-10]. Degradation of ROS depends on different enzymatic and non-enzymatic systems present in sperm and seminal plasma [11, 12]. In human and equine seminal plasma, superoxide dismutase, catalase and glutathione peroxidase have been described as the primary enzymatic antioxidants, while the exact contribution of non-enzymatic antioxidants to the total antioxidant capacity has not yet been established [13-17]. On the other hand, intrinsic sperm antioxidant defences include enzymes (such as paraoxonase, thioredoxin and the peroxiredoxin families of proteins) and glutathione (GSH), the most important non enzymatic antioxidant [11, 18-20]. Stallion sperm are characterized by higher GSH levels compared to other mammals and a significant correlation between the thiol content and stallion sperm functionality has been reported [5, 21, 22]. However, intrinsic sperm antioxidant defences, due to the limited amount of cytoplasm, offers only little protection. These sperm antioxidant defences can be rapidly overwhelmed in conditions of oxidative stress such as those occurring during sperm cryopreservation procedures that expose sperm to physical and chemical stressors that generate an increase of ROS, while seminal plasma, endowed with high radical scavenging activity, is preventively removed by centrifugation from the sample. For this reason, the addition of several antioxidants to sperm freezing media has been tested in different species in order to mitigate the adverse effects of ROS during cryopreservation and to better maintain frozen-thawed sperm quality and function [23-26]. Several studies demonstrated that a further approach is to supplement semen extender with antioxidant compounds during thawing; post-thaw treatment of boar sperm with resveratrol and epigallocatechin-3-gallate, alone or in combination, has been

demonstrated to improve *in vitro* fertilization (IVF) outcome [25, 27] while MnTBAP (a synthetic metalloporphyrin) exerts a positive effect on sperm motility, viability, lipid peroxidation and DNA integrity of frozen-thawed stallion sperm [28].

Recently, the incubation of frozen-thawed stallion sperm with cystine (CysS) has been demonstrated to induce an increase of intracellular GSH content and of total antioxidant capacity of frozen-thawed stallion sperm. In fact, extracellular CysS is exchanged for intracellular glutamate via the SLC7A11 antiporter, recently discovered in stallion sperm [29] and, once in the cell, CysS is reduced to cysteine (Cys) and used to synthesize GSH that exerts a fundamental role in maintaining redox homeostasis in sperm [30-33]. Moreover, Cys/Cyss redox node has been suggested to be functional in stallion sperm and CysS may provide antioxidant capacity independently of its incorporation into GSH [29].

Cryodamage has been described to induce structural modifications in several proteins, such as membrane transporters [34], and it has been suggested that SLC7A11 antiporter may present altered functionality in stallion cryopreserved sperm [29].

On these bases, this study was aimed at investigating the influence of CysS and the role of SLC7A11 antiporter on frozen-thawed stallion sperm ability to respond to *in vitro* capacitating environment after post-thaw incubation with CysS and/or Sulfasalazine (SS), a specific inhibitor of SLC7A11 antiporter. Sperm viability, motility, immunolocalization of tyrosine phosphorylated proteins, and the ability to bind to heterologous zonae pellucidae were evaluated.

2. Materials and Methods

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (Milan, Italy).

2.1. Semen processing

Frozen-thawed semen (two ejaculates from seven different stallions of proven fertility) housed at the National Institute of Artificial Insemination (AUB-INFA), University of Bologna, were used. Straws were thawed in a water bath at 37°C for at least 30 seconds and then diluted (v/v) in pre-warmed

Tyrodes medium pH 7.4 (20mM HEPES, 5mM Glucose, 96mM NaCl, 15mM $NaHCO_3$, 1mM Na-Pyruvate, 21.6 Na-Lactate, 2mM $CaCl_2*2H_2O$, 3.1mM KCl, 0.4mM $MgSO_4*7H_2O$, 0.3mM $NaH_2PO_4*H_2O$, 0.3% BSA). Samples were centrifuged (600g × 10') and resuspended in Tyrodes medium to a final concentration of $50x10^6$ sperm/mL.

2.2. Experimental design

Each frozen-thawed ejaculate was divided in Control (CTR) and 3 samples supplemented with: 0.5mM Cystine (CysS), 500µM Sulfasalazine (SS), a specific inhibitor of SLC7A11 antiporter, and 0.5mM CysS + 500µM SS (CysS+SS) and incubated in a water bath at 37°C for 1h. CysS and SS concentrations have been chosen based on previous studies [29].

Samples were then washed twice in Biggers, Whitter and Whittingham (BWW) medium pH 7.4 (20mM HEPES, 5.6mM D-Glucose, 95mM NaCl, 25mM $NaHCO_3$, 0.275mM Na-Pyruvate, 3.7µL/mL Sodium Lactate 60% w/v, 1.7mM $CaCl_2*2H_2O$, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4*7H_2O$, 0.1% PVA) and resuspended in a capacitating BWW medium (BWW supplemented with 5mM Dibutyryl cyclic AMP, 0.5mM Methyl-β-cyclodextrin and 3mM Caffeine) to a final concentration of $10x10^6$ sperm/mL and incubated at 38°C in 95% humidity and 5% CO_2 in air, as described by Bromfield et al., 2014 [35].

After 30 min of capacitation, $4x10^5$ sperm were co-incubated with 20 denuded pig oocytes for the heterologous binding assay.

Sperm viability, motility and tyrosine phosphorylated protein immunolocalization were evaluated in each sample after 1h of incubation in Tyrodes medium and after 30 min and 1h of incubation in capacitating condition.

2.3. Sperm viability assessment

Twenty-five microliters of semen were incubated with 2µL of a 300µM propidium iodide (PI) solution and 2µL of a 10µM SYBR green-14 solution, both from the live/dead sperm viability kit (Molecular Probes, Inc., Eugene, OR, USA), for 5 min at 37°C in the dark. Aliquots of the stained suspensions were placed on clean microscope slides and carefully overlaid with coverslips, and at least 200 sperm per sample were scored under a Nikon Eclipse E 600 epifluorescence microscope (Nikon

Europe BV, Badhoevedorp, The Netherlands). Sperm stained with SYBR-14 and not stained with PI were considered as viable. Sperm both SYBR-14+ and PI+ and those only stained with PI were considered dead.

2.4. Sperm motility assessment

Sperm motility was measured by a computer-assisted sperm analysis system, using the open source Image J BGM plugin as described by Giaretta et al., 2014 [36]. Briefly, a Leitz diaplan microscope (Wild LeitzGmbH, D6330, Wetzlar, Germany) with a 10x plan objective with negative phase-contrast was used. The microscope was equipped with a Z31 Ascon technologic heated stage (Ascon technologic, PV–IT). The video camera, a 3.1-megapixel CMOS USB 2.0 Infinity 1-3 Camera (Lumenera corporation, Ottawa, ON, Canada), was coupled with the microscope using a c-mount adapter and videos were recorded for three seconds at a resolution of 800×600 pixels and 60 frames/sec (fps). Images were saved on a hard drive using the Infinity analysing and capture software 6.4 (Lumenera corporation) and converted to avi format for subsequent analysis using the BGM Image J plugin.

Prior to any observation, sperm were loaded on a fixed height Leja Chamber SC 20-01-04-B (Leja, CIUDAD; The Netherlands). Five videos of separate fields, each lasting three seconds, were recorded per sperm sample and the central second of each video was analysed. Sperm motility endpoints assessed were: percent of total motile sperm ©, percent of progressive sperm (PM), curvilinear velocity (VCL) and mean velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), beat cross frequency (BCF), lateral head displacement (ALH) and wobble (WOB). The setting parameters used by the program were the following: 60 frames per second, number of frames 45, threshold path minimum; minimum VAP for motility 20µm/second; minimum VCL for motility 30µm/second; VAP cut off for progressive cells 15µm/second; STR cut off for progressive cells 45%.

2.5. Immunolocalization of tyrosine phosphorylated proteins

Samples of each experimental group, after 1h of incubation in Tyrodes medium and at 30 min and 1h of capacitation, were washed twice in PBS and aliquots of 80µL were placed on poly-l-lysine-coated slides and fixed with cold methanol for 15 min at −20°C and then with acetone for 30

seconds. Slides were washed with PBS, allowed to dry and stored at −20°C until staining. Unspecific bindings were blocked with PBS with 10% foetal calf serum (FCS) for 1h at room temperature in the dark. Analysis of tyrosine phosphorylation status was performed with an anti-phosphotyrosine antibody (1:150 clone 4G10, Millipore, Italy) incubated overnight at 4°C. After three washes in PBS, slides were incubated with a sheep anti-mouse IgG FITC conjugated (1:850, BioFx, Italy) for 60 min at room temperature in the dark. Slides were washed three times in PBS and mounted with a drop of Vectashield (Vector Laboratories, Burlingame, CA, USA). Oil immersion at 400× magnification was used utilizing the microscope described above, and at least 200 cells per sample were scored. For negative control samples, the same treatment was applied, omitting the primary antibody.

2.6. Heterologous binding assay

2.6.1. Pig oocyte *in vitro* maturation (IVM)

Ovaries were collected from pre-pubertal gilts at a local slaughterhouse and transported (in 0.9% wt/vol NaCl solution) to the laboratory within 2 h. Cumulus–oocyte complexes (COCs) were aspirated from antral follicles, 3-6 mm in diameter, with a 18-gauge needle fixed to a 10-mL disposable syringe. Under a stereomicroscope, intact COCs were selected and transferred into a petri dish (35 mm, Nunclon, Denmark) prefilled with 2mL of modified PBS supplemented with 0.4% BSA. After three washes in NCSU 37 supplemented with 5µg/mL insulin, 1mM glutamine, 0.57mM cysteine, 10ng/mL epidermal growth factor (EGF), 50µM ß-mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500µL of the same medium per well and cultured at 39°C in a humidified atmosphere of 5% CO_2 in air [37]. For the first 22 hours of *in vitro* maturation the medium was supplemented with 1mM Dibutyryl cyclic AMP and 0.12 IU/mL Pluset (CALIER, Italia); for the last 22 hours, COCs were transferred to fresh maturation medium [38]. At the end of the maturation period, the oocytes were denuded by gentle repeated pipetting, transferred in hypertonic solution (1.5M $MgCl_2$, 40mM HEPES, 0.1% PVP) and stored at 4°C until use. Before use, stored pig oocytes were washed twice in PBS.

2.6.2. Heterologous binding assay

For the binding assay, control semen and semen previously treated with CysS, SS or CysS+SS and then incubated for 30 min in capacitating condition, were co-incubated ($1x10^6$ sperm/mL) with 20 denuded oocytes in 400µL of capacitating BWW medium at 38°C in 95% humidity and 5% CO_2 in air. After 30 minutes of gamete co-incubation, the oocytes were washed four times in PBS 0.4% BSA with a wide bore glass pipette to remove the sperm loosely attached to the zonae pellucidae (ZP). The oocytes were then incubated with 8.9µM Hoechst 33342 for 10 min in PBS 0.4% BSA in the dark, washed twice in the same medium, and individually placed in droplets of Vectashield (Vector Laboratories) on a slide and covered with a coverslip. The number of sperm attached to the ZP of each oocyte was assessed using the above-described microscope. A total of 1000 oocytes were evaluated (240 for CTR, 255 for CysS, 262 for SS and 243 for CysS+SS).

2.7. Statistical analysis

Statistical analyses were performed using R (version 3.5.2). Values are expressed as mean ± SEM, unless otherwise specified and the level of significance used was $p \leq 0.05$. Motility and post thawing parameters were tested for normality and homogeneity of variances through Shapiro-Wilk and Levene tests. Then, a mixed effect model was set to determine treatment and time effects (1h post-incubation in Tyrodes medium, 30 min post-capacitation and 1h post-capacitation) and their interaction, with the ejaculate and repetition as random effects.

As for oocyte binding assays, the results are expressed as the number of sperm bound per oocyte normalized to the daily standard deviation. The variable was analysed using a general linear model with Poisson distribution and a Tukey post-hoc test was subsequently run to determine differences between treatments.

3. Results

3.1. Sperm viability and motility assessment

No significant differences were found in viability and motility parameters (TM, PM, VCL, VAP, VSL, STR, LIN, BCF ALH, and WOB) among experimental groups after incubation in Tyrodes medium (Fig.

1) (Supplementary file 1). Sperm incubation in capacitating condition induced a significant decrease in viability, total motility and progressive motility independently form the experimental group (*p<0.05*) (Fig. 1).

3.2. Detection of tyrosine phosphorylated proteins

Different patterns were detected as described by Balao da Silva et al., 2013 [39]. In this study, sperm with immunoreactivity either on the tail or on the tail and head (equatorial region and/or acrosome) were classified as capacitated (Fig. 2).

A significant increase in the percentage of immunoreactive sperm for tyrosine-phosphorylated proteins was observed after 30 min of capacitation (*p<0.05*) in all the experimental groups with the exception of CysS group, in which a non-significant tendency to an increase of sperm tyrosine-phosphorylation was observed (Fig. 3).

After 1h in capacitating condition the percentage of immunoreactive cells in each experimental group decreased to levels similar to those observed after Tyrodes incubation (Fig. 3).

No significant differences in the percentage of positive cells were recorded among the different experimental groups at the same time points of the experiment.

3.3. Heterologous binding assay

No significant difference was observed between the number of sperm bound per oocyte (mean ± SEM) in CTR and CysS groups (40.8 ± 10.8 *vs* 44.0 ± 12.3). The inhibition of SCL7A11 antiporter with SS (SS and CysS+SS experimental groups) significantly decreased (*p<0.001*) the number of sperm bound to the ZP compared to CTR and CysS groups (Fig. 4).

4. Discussion

In this study, the effect of CysS addition and the functionality of SLC7A11 antiporter on frozen-thawed stallion sperm ability to respond to *in vitro* capacitating environment was investigated.

Recently, Ortiz-Rodriguez et al. have described SLC7A11 presence and activity in stallion sperm and they have demonstrated that supplementing CysS, which in the cell is reduced to Cys and used in GSH synthesis, lead to an increased intracellular GSH concentration; this effect was prevented by SS, the specific SLC7A11 inhibitor [29, 30].

The results obtained from the evaluation of frozen-thawed stallion sperm viability and motility did not evidence any significant effect due to either the addition of CysS or the inhibition of SLC7A11 transporter, both in presence and in absence of CysS, at the end of 1h post-thaw incubation in Tyrodes medium. It is well known that sperm cryopreservation induces alterations in membrane stability and integrity [40, 41]. In agreement with other studies on frozen-thawed semen, which recorded a viability after thawing around of 40-50% in stallions that have good freezability [42, 43], the percentage of live cells recorded in this study after thawing semen for each stallion was around 40%, without differences between experimental groups. Nevertheless, it cannot be excluded that the incubation time of 1h with CysS and SS may be too short to induce evident modification in sperm motility and viability or that the functionality of SLC7A11 antiporter may be partially altered in cryopreserved spermatozoa as previously suggested by Ortiz-Rodriguez et al [29].

A significant decrease in viability, total motility and progressive motility was recorded when frozen-thawed sperm of all experimental groups were incubated in capacitating condition. A reduction in sperm viability associated with in vitro sperm capacitation process has been described in numerous studies [28, 44]. Capacitation is a process that sperm must undergo in order to fertilize the oocyte, requiring large quantities of energy in the form of adenosine triphosphate (ATP), which is synthesized during OXPHOS in the mitochondria. OXPHOS is responsible for ROS production and these molecules, at very low and controlled concentrations, have been recognized to participate in signal transduction mechanisms involved in sperm activation and capacitation [7, 45-48]. Sperm capacitation, the process that sperm must undergo in order to fertilize the oocyte, is characterized by increase in membrane fluidity, cholesterol depletion and lipid raft aggregation of the sperm plasma membrane, in addition to protein tyrosine phosphorylation [46]. Therefore, the decrease in sperm viability recorded when samples have been *in vitro* capacitated may be due to membrane instability induced by the capacitating events. Moreover, it has been reported that a subpopulation of cryopreserved sperm shows capacitation-like changes/characteristics after thawing, since

cryopreservation induces damages of the sperm plasma membrane, producing spermptosis [22, 49-51]. On these bases, the reduction of sperm viability and motility recorded after incubation of frozen-thawed stallion sperm in capacitation conditions may be due to a high sensitivity of cryopreserved sperm to capacitating stimuli that explains their limited life span and the loss of functionality as observed in small ruminants [52, 53]. Anyway, the addition of CysS, SS or a combination of CysS and SS did not induce any difference in sperm viability and motility between experimental groups under capacitation conditions suggesting that the effect of CysS and SS observed on oocyte binding ability is not due to an impact on those two sperm parameter. Phosphorylation of tyrosine residues on sperm proteins is considered a suitable marker of capacitation. As previously described in the equine spermatozoa [39, 54], we observed three main patterns of phospho-tyrosine immunopositivity on stallion sperm: tail, tail and head, or none. As previously mentioned, cryopreservation can induce capacitation-like changes after thawing, so it was not surprising to observe that, after 1h post-thawing incubation in Tyrodes prior to induction of capacitation, in all the experimental groups a subpopulation of sperm displayed tyrosine phosphorylation immunoreactivity. After incubation in capacitating condition, all experimental groups showed a significant increase in the percentage of sperm displaying protein tyrosine phosphorylation, with the exception of CysS group that showed only a not significant tendency towards higher levels of protein tyrosine phosphorylation. As already mentioned, CysS enters the cytosol of stallion sperm through the SLC7A11 antiporter and is used for the synthesis of GSH that plays an important role as an intrinsic spermatic antioxidant, causing a decrease of sperm ROS levels [11, 29]. Protein tyrosine phosphorylation is also a redox-regulated process [45, 55, 56] as ROS inactivates tyrosine phosphatases (PTPs) permitting the up-regulation of tyrosine phosphorylation that characterizes sperm capacitation [53]. Therefore, it can be hypothesized that the lower increase in protein tyrosine phosphorylation in the CysS experimental group we observed after *in vitro* induction of capacitation, may be due to the increase in intracellular GSH induced by CysS supplementation, as described by Ortiz-Rodriguez et al. [29], that may have induced a decrease in ROS levels and in turn a lower inhibition PTPs.

In this study, the influence of CysS and SLC7A11 functionality on stallion sperm ability to interact with oocyte ZP has been tested using heterologous ZP binding assay. This assay has been developed

to address difficulties associated with the acquisition of horse oocytes [45, 57, 58] and was demonstrated to be as reliable as homologous one [44]. The supplementation of CysS during 1h post-thaw of incubation in Tyrodes was unable to increase ZP binding after *in vitro* induction of capacitation. Interestingly, both the experimental groups in which SLC7A11 antiporters were inhibited by SS (SS and CysS+SS) showed a decrease in the number of sperm bound per oocyte. The inhibitory effect exerted by SS on sperm binding ability firstly suggest that the incorporation of CysS by SLC7A11 may exert a role in allowing sperm acquisition to interact with oocyte ZP after capacitation and, secondly, suggest that SLC7A11 functionality is maintained, at least in part, in frozen-thawed stallion sperm.

As previously mentioned, CysS incorporated into the sperm is used to synthetize GSH which is implicated in processes such as neutralization of superoxide and detoxification of metabolites [59-61]. GSH can also interact with its associated enzymes (glutathione peroxidases, glutathione reductases and glutathione S-transferases), to provide protection against oxidative stress. It has been reported that sperm glutathione S-transferases play an important role in fertilization, as specific glutathione S-transferase members are involved in sperm-oocyte binding [62, 63]. It can be hypothesized that the reduction of CysS incorporation induced by SLC7A11 inhibition carried out by SS may have affected sperm-ZP binding through an impairment of glutathione S-transferases function.

In conclusion, the incorporation of CysS through the SLC7A11 antiporter in frozen-thawed stallion sperm may influence the sperms ability to bind to heterologous zonae pellucidae. The mechanism by which this effect is exerted remains to be elucidated as sperm motility, membrane integrity and protein tyrosine phosphorylation were not affected.

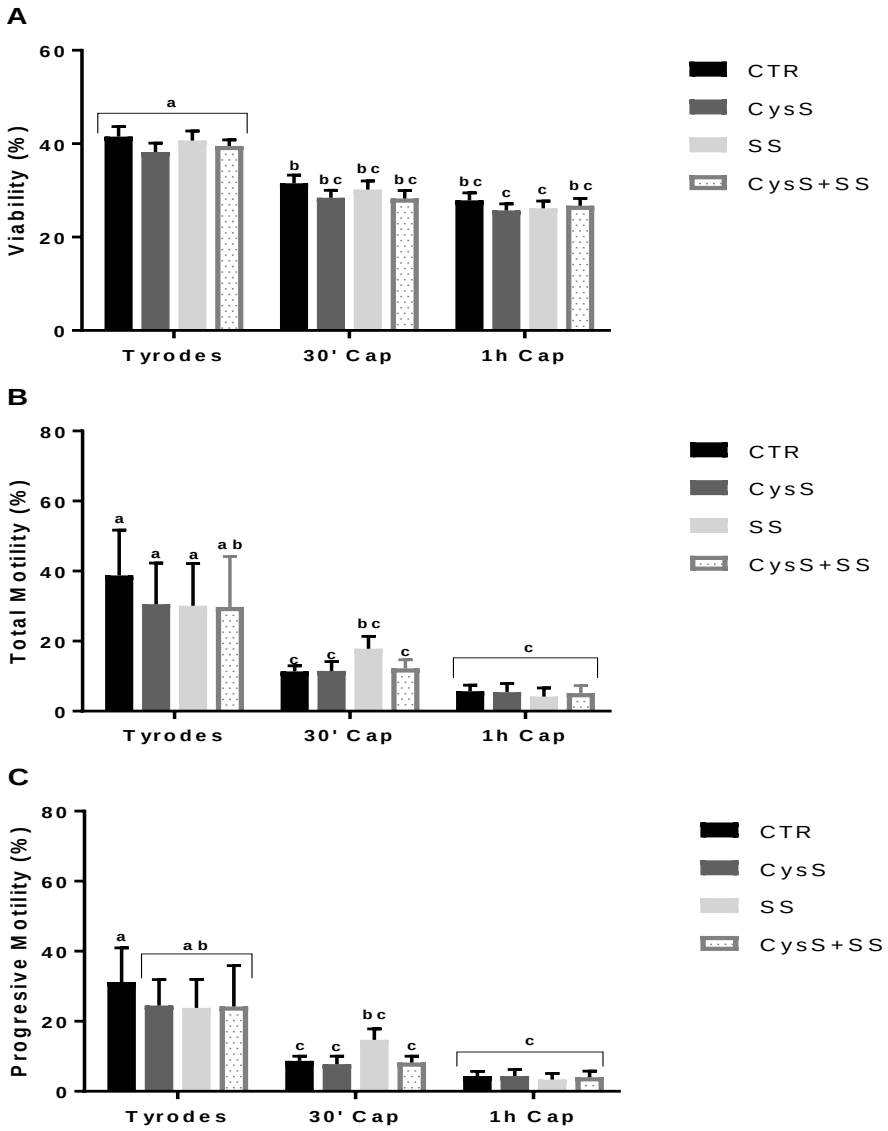


Fig. 1. Effect of 0.5mM CysS, 500µM SS and 0.5mM CysS + 500µM SS supplementation during post-thaw incubation at 37°C for 1h on Viability (A), Total Motility (B) and Progressive Motility (C) of frozen-thawed stallion sperm, before (Tyrodes) and after 30 min (30' Cap) and 1h (1h Cap) under capacitating condition. Different letters indicate a significant difference between incubation times ($p<0.05$). Data represent the mean ± SEM.

FIGURE 2 CAPTION (UPLOADED AS SEPARATE FILE)

Fig. 2. Representative micrographs of protein tyrosine immunostaining (green). (A) Negative (left) and positive spermatozoon (right; tail positivity); (B) Negative (upward) and positive spermatozoon (downward; tail and head positivity).

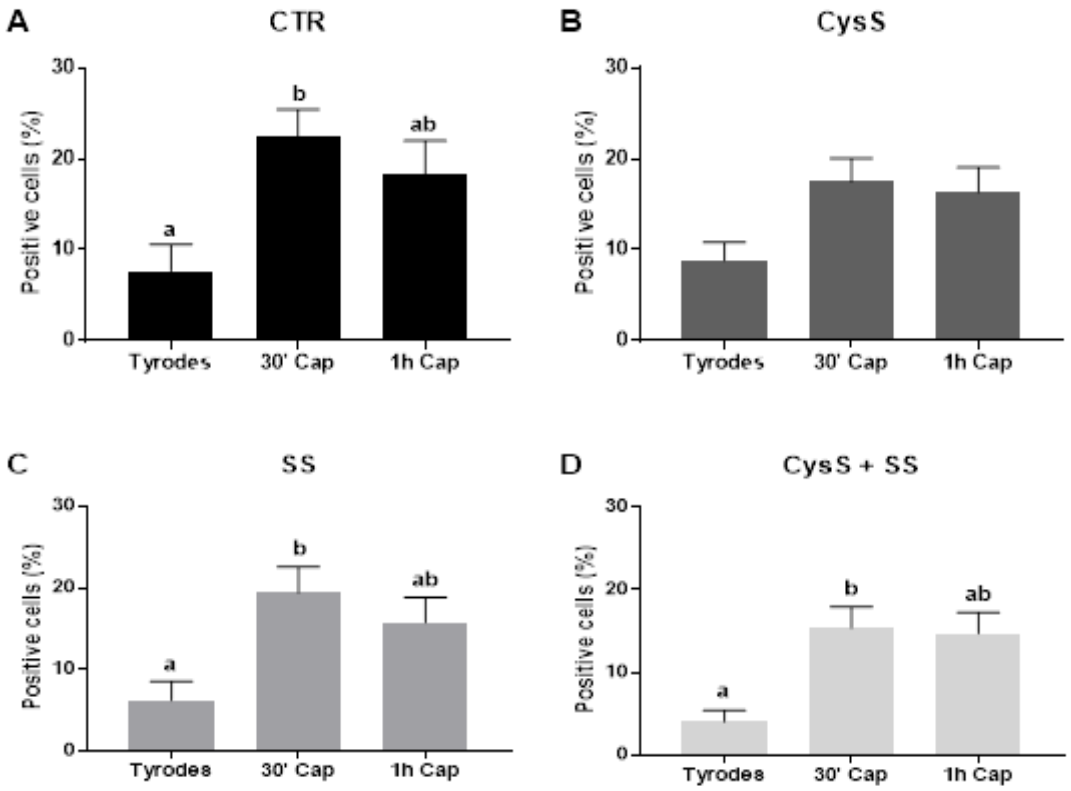
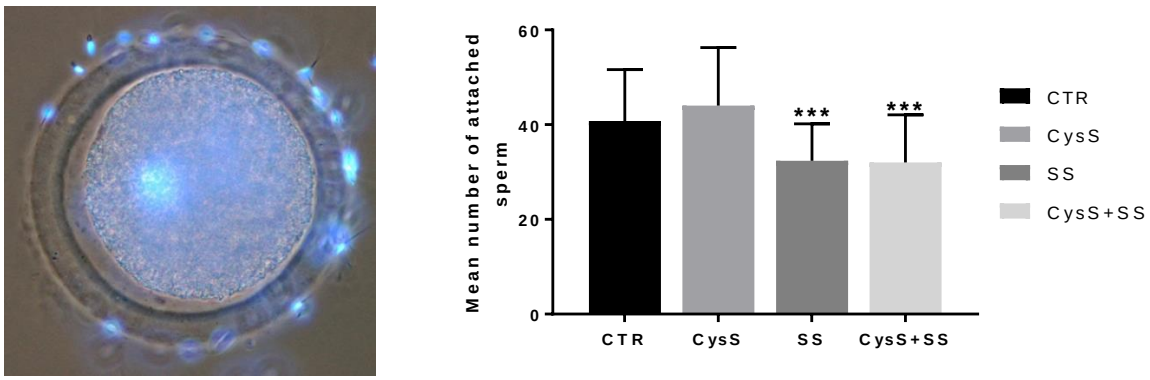


Fig. 3. Percentages of cells displaying tyrosine phosphorylation immunoreactivity before (Tyrodes) and after incubation in capacitating condition for 30 min (30' Cap) and 1h (1h Cap) in control group (A) and in groups supplemented during post-thaw incubation at 37°C for 1h with 0.5mM CysS (B), 500µM SS (C) and 0.5mM CysS + 500µM SS (D). Different letters represent a significant difference for *p<0.05* in the same treatment between incubation times. Data represent the mean ± SEM.



A **B**

Fig. 4. (A) Epifluorescence and phase contrast microscopy overlapped images of a pig oocyte with bound stallion sperm stained with Hoechst 33342. (B) Effect of CysS (0.5mM), SS (500µM) and CysS+SS (0.5mM CysS + 500µM SS) supplementation during post-thaw incubation at 37°C for 1h on the number of bound sperm per porcine oocyte after incubation in capacitating condition. A total of 1000 oocytes were evaluated (numbers of oocytes analyzed in the different groups were: 240 for CTR, 255 for CysS, 262 for SS and 243 for CysS+SS).****p<0.001*. Data represent the mean ± SEM.

Supplementary file 1

Effect of 0.5mM CysS, 500µM SS and 0.5mM CysS + 500µM SS supplementation during post-thaw incubation at 37°C for 1h on sperm motility parameters of frozen-thawed stallion sperm, before (Tyrodes) and after 30 min (30' Cap) and 1h (1h Cap) under capacitating condition. VCL, curvilinear velocity (µm/sec); VAP, average path velocity (µm/sec); VSL, straight line velocity (µm/sec); STR, straightness (%); LIN, linearity (%); WOB, wobble (%); BCF, beat cross frequency (Hz); ALH, amplitude of lateral head displacement (µm). Data represent the mean ± SEM.

	CTR Tyrodes	CysS Tyrodes	SS Tyrodes	CysS+SS Tyrodes
VCL (µm/s)	140,81 ± 11,16	136,59 ± 10,04	140,33 ± 8,33	132,74 ± 10,52
VAP (µm/s)	59,71 ± 5,18	55,75 ± 4,97	60,86 ± 4,72	55,66 ± 4,88
VSL (µm/s)	40,32 ± 3,63	36,29 ± 3,54	40,81 ± 3,14	37,96 ± 3,16
STR (%)	49,26 ± 8,58	48,76 ± 8,48	49,09 ± 8,51	49,93 ± 8,67
LIN (%)	26,01 ± 4,72	24,48 ± 4,42	25,38 ± 4,45	25,22 ± 4,48
WOB (%)	32,30 ± 5,82	31,93 ± 5,63	31,65 ± 5,55	30,99 ± 5,44
BCF (Hz)	25,18 ± 0,42	25,12 ± 0,36	24,79 ± 0,25	25,38 ± 0,33
ALH (µm)	5,27 ± 0,40	4,98 ± 0,37	5,31 ± 0,31	4,85 ± 0,40

	CTR 30' Cap	CysS 30' Cap	SS 30' Cap	CysS+SS 30' Cap
VCL (µm/s)	127,56 ± 18,18	157,45 ± 11,91	121,05 ± 11,31	141,78 ± 13,71
VAP (µm/s)	44,94 ± 3,66	48,92 ± 2,41	45,84 ± 3,81	47,22 ± 3,46
VSL (µm/s)	29,08 ± 1,65	27,95 ± 2,31	30,70 ± 2,90	27,08 ± 1,97
STR (%)	52,84 ± 9,34	43,51 ± 8,05	51,88 ± 9,25	44,21 ± 8,47
LIN (%)	32,66 ± 6,10	22,70 ± 5,17	29,90 ± 6,36	23,75 ± 6,69
WOB (%)	34,67 ± 6,42	27,42 5,49	31,69 ± 6,11	30,33 ± 6,93
BCF (Hz)	25,95 ± 0,69	23,80 ± 0,54	24,70 ± 0,41	24,74 ± 0,51
ALH (µm)	4,59 ± 0,63	5,62 ± 0,36	4,50 ± 0,36	4,99 ± 0,49

	CTR 1h Cap	CysS 1h Cap	SS 1h Cap	CysS+SS 1h Cap
VCL (µm/s)	127,77 ± 15,06	167,86 ± 15,52	133,91 ± 12,52	119,77 ± 16,53
VAP (µm/s)	53,10 ± 4,48	56,63 ± 5,36	50,26 ± 3,93	46,56 ± 3,46
VSL (µm/s)	35,98 ± 3,47	29,17 ± 1,95	32,04 ± 4,04	32,09 ± 3,16
STR (%)	49,72 ± 9,12	43,11 ± 8,10	50,95 ± 9,64	51,94 ± 9,77
LIN (%)	31,52 ± 6,40	22,49 ± 5,49	31,47 ± 7,04	34,99 ± 8,06
WOB (%)	33,61 ± 6,34	27,34 ± 5,39	36,39 ± 7,25	37,13 ± 8,14
BCF (Hz)	23,92 ± 0,76	25,44 ± 0,93	23,85 ± 0,80	24,64 ± 1,09
ALH (µm)	5,16 ± 0,68	5,69 ± 0,52	4,44 ± 0,40	4,84 ± 0,37

Authors contribution:

OR JM, MS and GG designed the work, conducted the experiments and wrote the manuscript. CN and BM conducted the experiments. DB conducted the experiments and performed the statistical analysis. CT and FP critically revised the work. All authors discussed the results and contributed to the final manuscript.

Declaration of interest

None of the authors have conflict of interest to declare.

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