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# Effect of sod (superoxide dismutase) protein supplementation in semen extenders on motility, viability, acrosome status and ERK (extracellular signal-regulated kinase) protein phosphorylation of chilled stallion spermatozoa

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**Abstract**

New studies are underway to find new methods for supporting longer storage of cooled stallion semen. It is known that high concentrations of reactive oxygen species (ROS) cause sperm pathology. The metalloprotein superoxide dismutase (SOD) is responsible for $H_2O_2$ and $O_2$ production, by dismutation of superoxide radicals. The aim of this study is to assess the quality of chilled stallion semen processed with extenders containing SOD at different concentrations as antioxidant additives. A total of 80 ejaculates collected from 5 standardbred stallions was divided into 5 aliquots treated as: native semen (control 1); native semen diluted 1:3 with Kenney semen extender (control 2); spermatozoa diluted after centrifugation in extender without (control 3) or with SOD at 25 IU/ml (experimental 1) or 50IU/ml (experimental 2). Each sample was analyzed for motility, viability and acrosome status, immediately after semen preparation and again after storage at 5 °C for 24h, 48h and 72h.

Acrosome integrity was evaluated by Chlortetracycline (CTC) and Fluorescent-labeled peanut lectin agglutinin (PNA-FITC conjugated staining). A proteomic approach of quantifying extracellular signal regulated kinase (ERK) was also evaluated as an indirect indicator of oxidative stress. In all samples sperm progressive motility and sperm acrosomal integrity showed a significant reduction between fresh and cooled spermatozoa at 24h, 48h and 72h. Quality parameters of sperm were significantly higher (Progressive Motility $P < 0.01$; Viability $P < 0.001$) in aliquots supplemented with SOD. ERK phosphorylation was statistically higher ($P < 0.01$) in aliquots without SOD. The Authors concluded that addition of SOD to semen extenders improves the quality of chilled equine semen and reduces ERK activation.


## 1. Introduction

Artificial insemination in equine species can be performed with fresh, cooled or frozen–thawed semen. A worldwide upsurge in the use of cooled stallion semen has occurred over the past decade and semen is couriered by air on a regular basis over very long distances. The shipment of semen saves large sums by avoiding transportation of mares to the stallion of choice and greatly broadens the range of stallions available to owners of mares. The fertility of cooled semen is maintained for 24–48 h. After that time, pregnancy rates

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decrease dramatically [1]. The cooled semen revolution has spawned the development of new extenders that act to ensure longer storage. In order to maintain fertilizing ability of semen as long as possible and reduce problems such as impaired semen quality after cooled storage, it is necessary to use appropriate extenders.

Not all stallions can be used for cooled-semen production because in some sires fertility decreases when their semen is processed, cooled and transported [1]. For this reason, one major factor affecting the longevity of equine spermatozoa during storage and shipping is the stallion itself. Stallions may be classified as "good" or "bad" coolers based on the suitability of their semen to be processed for cooled-shipping. This not only depends on the quality of native semen, but also on the composition of the seminal plasma and sperm plasma membranes [1].

Recent findings in equine reproductive technology have increased the quality and fertility of cooled semen and horse breeding benefits from this biotechnology [1]. In order to maintain semen fertilizing ability for a longer period, appropriate extenders must be used and cooled semen must be kept at 4 °C; even so, extended cooled semen should be used within 72 h after collection [2].

All aerobic organisms require oxygen for life. Although it is an essential element, oxygen is responsible for reactive oxygen species (ROS) production. A review by Medeiros et al [3] notes that the ROS-generating mechanism of spermatozoa has not been characterized. Electron leakage from the sperm mitochondria is thought to constitute the major source of reactive oxygen species (ROS) in these cells [4]. Low, controlled concentrations of ROS play an important role in sperm physiology while higher concentrations are detrimental. A Study by O'Flaherty et al [5] examined the influence of ROS on capacitation and the acrosome reaction in frozen-thawed bull spermatozoa; they concluded that ROS is required in the capacitation process and that hydrogen peroxide may participate as an inducer of the acrosome reaction [6].

Reactive Oxygen Species behave as second messengers, regulate sperm capacitation, induce the acrosome reaction and support oocyte fertilization. They regulate downstream events: first, the increase of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) activation and phosphorylation of PKA substrates (arginine-X-X-serine/threonine motif); second, the phosphorylation of extracellular signal regulated kinase [ERK] kinase-like (MEK) proteins and then that of the threonine-glutamate-tyrosine motif; and finally, the late tyrosine phosphorylation of fibrous sheath proteins [7]. Therefore, while critically important, ROS concentrations should be limited to the minimum required to maintain normal cell functions [6].

When the production of ROS by sperm mitochondria is excessive, then the gamete's limited endogenous antioxidant defences are rapidly overwhelmed and oxidative damage is induced [8]. High concentrations of ROS are able to adversely change sperm cell functions, by ultimately endangering cell survival with deleterious alterations in fluidity and integrity of sperm membranes because of lipid peroxidation. Reactive Oxygen Species can also induce DNA damage in the sperm nucleus, deplete Adenosine tri-phosphate (ATP) in mitochondria, and cause loss of sperm motility, viability and capacity for fertilization [8,9].

A recent commentary on semen quality [10] notes that an important mechanism by which DNA damage is induced in the male gamete is oxidative stress; spermatozoa are particularly vulnerable to this because they generate ROS and are rich in targets for oxidative attack. The authors also draw attention to the fact that, because spermatozoa are transcriptionally inactive and have little cytoplasm, they are deficient in both antioxidants and DNA-repair systems [8]. Oxidative stress may be a cause of male infertility and contribute to DNA fragmentation in spermatozoa [8]. There are few studies on the effects of antioxidant addition to extenders during cooling and/or freezing mammalian spermatozoa [11]. In stallion semen, ROS are generated mainly by damaged and abnormal spermatozoa and by contaminating leukocytes. Reactive Oxygen Species damage cells by changes to lipids, proteins and DNA. It was suggested that peroxidative stress triggers the mitogen activated protein kinase (MAPKs) cascade and the extracellular signal-regulated protein kinase (ERK) phosphorylation [12]. Spermatozoa are potentially susceptible to peroxidative damage caused by ROS excess due to high amounts of polyunsaturated fatty acids in membrane phospholipids and to sparse cytoplasm.

Antioxidative systems control the balance between production and neutralization of ROS and protect spermatozoa against peroxidative damage [13]. Recent studies also show that there is physiological SOD activity in human seminal plasma [14,15].

The effects of ROS are prevented or diminished by the detoxifying enzymes Superoxide Dismutase (SOD), Catalase, Peroxidases and reducing agents such as Glutathione, Ascorbic acid, taurine and hypotaurine, all present in sperm cells and in their microenviroments. Several studies have evaluated the effects of different

antioxidants on extended mammalian semen [7,16,17,18]. The metalloproteins SOD are an important antioxidant defense in nearly all cells exposed to oxygen. Their use as additives in semen extenders has had controversial effects [17,18]. Previous studies demonstrate that equine spermatozoa produce ROS [17,19]. It was reported [20] that there is an equine semen SOD activity: mean glutathione peroxidase (GPX) and SOD-like activities in seminal plasma were 1.3 ± 0.1 nmol of NADPH oxidized/min/mg of protein and 29.2 ± 6.6 U/mg of protein, respectively. The mean GPX activities in spermatozoa separated from seminal plasma by centrifugation and via Percoll gradient were 2.2 ± 0.3 nmol and 6.1 ± 1.3 nmol of NADPH oxidized/min/mg of protein, respectively. Although the mean SOD-like activity of spermatozoa separated by centrifugation was 58.6 ± 22.3 U/mg of protein, SOD-like activity was not detected in Percoll-separated spermatozoa [20]. Another study reports that storage of extended stallion semen for 24 h at 5 °C does not change the activity of sperm enzymes. It may be that lipid peroxidation occurs over time and that addition of extenders has a positive effect on the antioxidant potential of semen [11].

Relatively little is known about the molecular antioxidant mechanisms in spermatozoa [21,22]. Mature spermatozoa are fully differentiated cells that lack active transcriptional machinery. Mitogen-activated protein kinase 3 (MAPK3) cascades may play a crucial role in cell cycle progression and apoptosis [6]. It has been demonstrated that ERK1/2 and Protein Kinase 38 (p38) MAPK are expressed in the tail of ejaculated human spermatozoa. Activation of ERK1/2 is downstream to Protein Kinase C (PKC) activation, and two of its isoforms, PKC and endogenus PKC inhibitor (PKC_I) are also present in the human sperm tail. An increased expression of total phosphorylated-ERK1 was reported to predict poor quality human spermatozoa and could be suggestive of oxidative stress. Inverse correlations exist between ERK1 and sperm motility, forward progression motility, sperm morphology and viability [17]. Hence, it is of great interest to investigate whether ejaculated spermatozoa express and use MAPKs during the oxidative stress response. It is possible to evaluate the efficiency of SOD addition to extenders indirectly by measuring the activity and phosphorylation of ERK.

The purpose of our study was to evaluate the effect of adding low concentrations of SOD to the extender on motility, viability, acrosome state and phosphorylation of ERK protein of equine spermatozoa during routine storage at +5 °C.

## 2. Materials and methods

### 2.1. Experimental animals

Five fertile standardbred stallions aged between 9 and 23 years with a body weight between 400 and 600 kg were available for the study. These stallions were housed in boxes, with access to an outdoor paddock from 7:00 to 16:00 h. They were fed with concentrate twice daily and with hay and water *ad libitum*.

### 2.2. Experimental design

Semen samples were collected from five stallions, two times a week for eight weeks, from 11 January 2010 until 5 March 2010, for a total of 16 samples/stallion and 80 total samples.

To reduce the variability of response to the addition of SOD in relation to sperm concentration and volume of residual seminal plasma, our study used only stallions that at the collection showed a similar concentrations of spermatozoa (mean ± SD 173 ± 8.4 × $10^6$ sperm/ml). In order to assess the effect of adding SOD at low concentrations to extenders in routine procedures for preparing semen for storage at +5 °C, each ejaculate was divided into five aliquots: (i) native semen as collected (Control 1); (ii) native semen diluted 1:3 (v:v) in Kenney semen extender (EZ-Mixin ® + ARS ®, CA) (Control 2); the three remaining aliquots were prepared as recently described [23] to optimize the semen storage at +5 °C: centrifugation at 1200 g for 10 min and removal of 75% of the supernatant seminal plasma. These three aliquots were then diluted to obtained a final concentration of 500 × $10^6$ sperm/ml in Kenney semen extender (iii) without SOD addition (Control 3) or with 25 IU/ml SOD (Experimental 1) or 50 IU / ml SOD (Experimental 2).

Enzymatic antioxidant activity in equine spermatozoa appears to be predominantly derived from seminal plasma adsorbed onto the plasma membrane. Removal of seminal plasma during equine semen processing may increase oxidative stress in spermatozoa [20]. Nevertheless, SOD was added only to the aliquots prepared as described above with 75% plasma removal [23] in order to assess the effect on semen stored at +5° with a routinely approved method, in view of the repeatedly demonstrated negative effects of seminal plasma during storage of equine semen at +5 °C [30].

Each of the five aliquots was subjected to chilling for 3, 24, 48 and 72 h. For each ejaculate and for each aliquot at each experimental time the following parameters of fertility were assessed: motility, viability and acrosome status. In addition to quantify the activity of SOD in reducing the negative effect of ROS in inducing the acrosome reaction cascade, we also evaluated the phosphorylation of ERK protein [12].

### 2.3. Semen collection

After a sexual rest of 1 week, semen was collected with a Missouri artificial vagina. For each ejaculate, the gel fraction was removed and semen was filtered through a sterile filter immediately after collection. Semen was examined by the general quality test [11], and parameters such as concentration (n $\times$ $10^6$ sperm/ml), progressive motility (%), viability (%) and acrosome integrity (%) were evaluated as described below.

### 2.4. Motility and viability test

Progressive motility was evaluated using a phase contrast microscope at 100 $\times$ magnification. The proportion of progressively motile spermatozoa from 8 randomly selected fields in each sample was evaluated subjectively in a Makler chamber at 37 °C. Sperm viability was evaluated before and after cooling at different times by Eosine staining (Viability stain–Europath, Naples, Italy). The sperm viability was also evaluated by using a nigrosine–eosine stain (NE) which was prepared as described by Tamuli and Watson [24]. The diluted sperm sample (5 $\mu$l) was mixed with the NE stain (10 $\mu$l) at 37 °C, incubated for 30 s, smeared and dried on a warm plate at 37 °C. The samples were evaluated using bright field microscopy at 400 $\times$ magnification. Live spermatozoa remained unstained, whereas dead cells were dull pink. The percentage of live spermatozoa was expressed as viability (%).

### 2.5. Acrosome status evaluation

Spermatozoa with normal acrosomes were identified using two different staining methods: fluorescent antibiotic chlortetracycline (CTC) (Sigma Aldrich, Milan, Italy, C4881) and fluorescently labeled peanut lectin agglutinin (PNA-FITC conjugated) (Vector Laboratories, FL, USA) [25]. A CTC solution was made with 5 ml of CTC buffer (TRIS solution: Hidroxymetilaminometane 3,634g, Fructose 0,50g, Citric Acid monohydrate 1,998g in water 100ml), 2 mg of CTC and 4.4 mg of cysteine. The sperm sample was overlaid with protein-free tissue culture medium 199 at a dilution of 3:1 (v/v) and centrifuged at 300 *g* for 20 min. The supernatant was removed and the pellet resuspended in Earle's medium supplemented with 4% of bovine serum albumin. A 45 $\mu$l volume of the CTC solution, 1 $\mu$g/ml propidium iodide and 8 $\mu$l of 12.5% (w/v) paraformaldehyde were added to 45 $\mu$l aliquots of each sperm sample and mixed. A 10 $\mu$l droplet of the fixed and stained sample of spermatozoa was placed on a microscope slide and mounted with a droplet of an aqueous mounting medium (Vectashield, Vector Laboratories, Peterborough, UK) to retard fading of the fluorescence. The CTC staining of the live spermatozoa was observed with a confocal laser scanning microscope LSM-510 (Zeiss, Gottingen, Germany). CTC was excited at 420 nm and detected via a 500 nm band-pass filter. Propidium iodide was excited at 488 nm and detected via a 560 long-pass filter. The different frames were scanned separately, with appropriate installation of the optical path for excitation and emission of each scan, according to previously published methods [26]. The typical features of CTC stained cells were observed, photographed and described. The three distinct patterns observed using the CTC staining were allocated according to an identification system as follows: F. uncapacitated and acrosome intact; B. capacitated and acrosome intact; AR capacitated and acrosome reacted [19,24]. To confirm the results obtained with this first staining method, the percentage of acrosome-reacted spermatozoa was determined microscopically on air-dried sperm smears using FITC-conjugated *Pisum sativum*. An aliquot of the sperm sample was spread on microscope slides and allowed to air dry. The spermatozoa were then permeabilized with methanol for 15 min at room temperature, washed once with 25 mM Tris-buffered saline, pH 7.6, for 5 min and then twice with $H_2O$ at 5 min intervals, air-dried and then incubated with FITC-*P. sativum* agglutinin (60 $\mu$g/ml) for 1 h, washed twice with $H_2O$ at 5 min intervals, and mounted with Vectashield. For each experiment, at least 100 cells per slide were evaluated. The patterns observed using the PNA agglutinin FITC-conjugated staining method have been described in different mammalian species and were characterized as acrosome intact (AI) or acrosome reacted (AR). Cells with green staining over the acrosomal cap were considered acrosome-intact; cells with equatorial green staining or no staining were considered acrosome-reacted.

### 2.6. Western Blot analysis

MAPK (ERK) activity was determined by Western blotting with type-specific antibodies for anti-ERK antibodies. Each band from the anti-phospho-ERK was

normalized to the corresponding band from the anti-ERK blot for equal loading [12] .

Sperm cells were precipitated by centrifugation at 1,500*g* for 10 minutes at room temperature, and washed once more. The pellets were stored at −20 °C until used. The next steps were done at 4 °C. The thawed pellets were resuspended in a minimal volume of lysis buffer (50$\mu$l per $3 \times 10^7$ cells) comprising 50 mM Tris-HCl, pH 8.0, 2 mM EGTA, 20 mM NaCl, 1.0 mM sodium orthovanadate, 25 mM $\beta$-glycerophosphate, 100 nM okadaic acid, 0.50% Nonidet P-40, 1mM benzamidine, 10$\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, 1mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol. After centrifugation at 10,000 *g* for 15 min at 4 °C, the protein concentration of the supernatant was determined by the Bradford assay. To measure ERK1/2, 30 $\mu$g of proteins were diluted in 5ml loading buffer (10 g/l SDS, 10% glycerol, 1% 2-mercaptoethanol, 5mM Tris-HCl, pH 6.8), boiled for 5 min, and separated on two 4–12% Sodium Dodecil Solfate–PolyAcrylamide Gel Electrophoresis (SDS-PAGE). After electrophoresis, the first gel was stained with Coomassie-blue: the gel was fixed in 25% Isopropyl Alchol (IPA) with 10% Acetic Acid (HoAc) in water for 60 minutes; The gel was stainedin 10% HoAC in water with 60mg/L of Comassie blue R-250, bands will appear in 30 min, finally destain gel in 10% HoAC for 2 h or more and store the gel in 7% HoC.The second gel was submitted to electro blotting to transfer the proteins onto a polyvinylidene difluoride membrane that was washed with phosphate-buffered saline (PBS). After washing, the membrane was incubated with blocking buffer (2 g/l highly purified casein, 1 g/l Tween 20 in PBS) for 1 h and then probed for 1 h with the antibody Anti-ERK1/2 (1:5000, Promega, USA). Secondary goat anti-rabbit IgG–IgM alkaline phosphatase conjugate was diluted 1:5.000 in 5ml of blocking buffer and added for 1 h, after two washes in 20 ml of blocking buffer. The last washing was performed three times with 20 ml of blocking buffer, and finally detection was obtained with a CSPD chemiluminescent substrate (Tropix, Bedford, MA). To test for equal loading of proteins (30 g), anti-actin antibody (Sigma Aldrich, Milan, Italy) diluted 1:1,000 or 0.5% Ponceau S in acetic acid was used. ERK1/2 were quantified by densitometric analysis and results were expressed as optical density.

### 2.7. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) performed using Graphpad in Stat Version for Windows XP and by Medcalc software (Frank Shoonjans, V.7.2.1.0.); comparisons between experimental groups means were performed with the Tukey-Kramer Multiple Comparisons test. Differences with values of $P < 0.05$ were considered to be statistically significant. Data are presented as mean ± standard error of the mean (SEM).

## 3. Results

Sixteen stallion ejaculates were collected and 80 total samples were analyzed. The ejaculates of the five stallions did not differ for fertility parameters tested at 24 h of storage. The sperm concentration of the samples

Table 1
Progressive motility (1a) and viability (1b) (Mean ± SEM) of the five tested aliquots of spermatozoa at the four different experimental times (1h, 24h, 48h, 72h).

| | 3 h | 24 h | 48 h | 72 h |
|---|---|---|---|---|
| Table 1a Motility | | | | |
| Native semen (Control 1) | 70 ± 6.8 | 35 ± 6.3$^{AC}$ | 20 ± 6.2$^{AC}$ | 5 ± 6.4$^{AC}$ |
| Extended semen (Control 2) | 68 ± 3.4 | 60 ± 2.5$^{AD}$ | 40 ± 6.6$^{AD}$ | 36 ± 3.2$^{AD}$ |
| Extended pellet (Control 3) | 74 ± 7.9 | 63 ± 2.1$^{AD}$ | 45 ± 3.1$^{AD}$ | 35 ± 2.1$^{AD}$ |
| SOD 25UI (Experimental 1) | 72 ± 5.3 | 70 ± 4.6$^{B}$ | 62 ± 2.7$^{B}$ | 47 ± 2.9$^{B}$ |
| SOD 50UI (Experimental 2) | 75 ± 2.6 | 70 ± 5.2$^{B}$ | 60 ± 2.5$^{B}$ | 45 ± 1.8$^{B}$ |
| $^{A–B, C–D}$ $P < 0.01$ (column). | | | | |
| Table 1b Viability | 3 h | 24 h | 48 h | 72 h |
| Native semen (Control 1) | 81 ± 5.2 | 47 ± 2.4$^{AC}$ | 32 ± 2.8$^{AC}$ | 15 ± 3.4$^{AC}$ |
| Extended semen (Control 2) | 80 ± 7.6 | 73 ± 6.7$^{AD}$ | 54 ± 4.2$^{AD}$ | 41 ± 7.2$^{AD}$ |
| Extended pellet (Control 3) | 83 ± 5.4 | 75 ± 5.2$^{AD}$ | 58 ± 2.9$^{AD}$ | 46 ± 3.1$^{AD}$ |
| SOD 25UI (Experimental 1) | 82 ± 7.6 | 81 ± 3.6$^{B}$ | 75 ± 3.4$^{B}$ | 58 ± 7.2$^{B}$ |
| SOD 50UI (Experimental 2) | 82 ± 2.5 | 79 ± 4.9$^{B}$ | 73 ± 2.6$^{B}$ | 55 ± 6.7$^{B}$ |
| $^{A–B, C–D}$ $P < 0.001$ (column). | | | | |

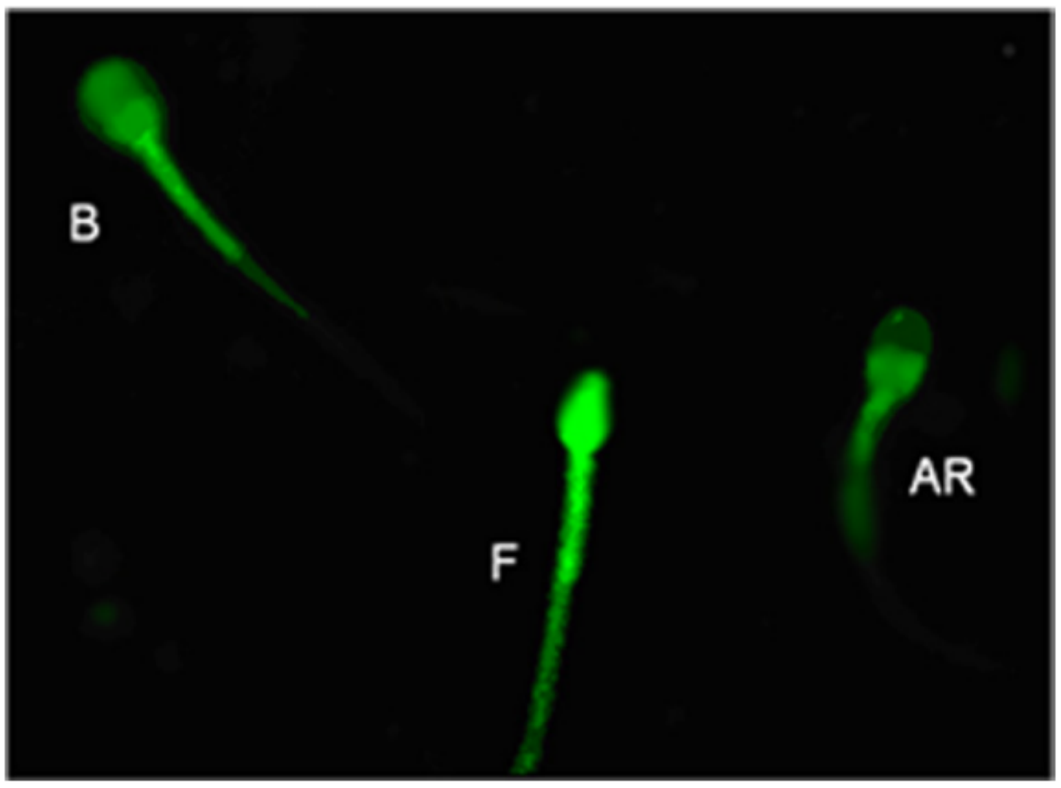


Fig. 1. Evaluation of acrosome status by CTC staining methods: The spermatozoa that show more intensely green fluorescence in the head equatorial region and acrosomal region are classified as undergoing acrosome reaction (B); spermatozoa showing a darker equatorial region and acrosomal region are classified as acrosome reacted (AR); spermatozoa showing a head without the equatorial region and totally very intensely green fluorescent are classified as acrosome intact (F).

ranged from 150 to 193 $\times$ $10^6$/ml (mean $\pm$ SD: 173 $\pm$ 8.4 $\times$ $10^6$/ml) and the progressive sperm motility at collection ranged from 57 to 78% (mean $\pm$ : 73 $\pm$ 6.3 %).

Means $\pm$ SEM for progressive motility and viability in fresh and in cooled stallion spermatozoa of the 5 different aliquots tested at the three different times are shown in Tables 1a and 1b, respectively. At 3 h of storage, there were no significant differences among treatments in any motility parameter. At 24 h, incubation with SOD was associated with a significant ($P < 0.01$) increase in motility compared with the control (Table 1a). Similar changes were also demonstrated at 48 and 72 h of storage. The addition of SOD at 25 and 50 UI/ml, in sperm extender significantly ($P < 0.001$) increased the percentage of viable spermatozoa (Table 1b). Differences in viability between control and experimental samples were significant at any times examined ($P < 0.001$).

Data from acrosomal status observed using the CTC (Fig. 1, Table 2 and 3) and PNA (Fig. 2a, 2b) staining methods showed that the addition of SOD in the control 1-2-3 and experimental 1-2 samples had no significant ($P > 0.05$) effect on the percentages of AI and AR cells.

Western blot analysis of ERK (Extracellular signal-regulated kinase) protein phosphorylation is shown in Table 4. The optical density of phosphorylated ERK 1 protein (p-ERK1) (Fig. 3) was significantly ($P < 0.01$) higher in control than in experimental aliquots at 3h, 24h and 48h of storage. The optical density of p-ERK1, after

Table 2
Non-capacitated and acrosome intact spermatozoa (Mean $\pm$ SEM) of fresh and cooled aliquots at different experimental times examined by CTC staining (F) and by FITC-conjugated Pisum sativum staining (AI).

| | Control 1(% $\pm$ SEM) | | Experimental 1(% $\pm$ SEM) | | Control 2(% $\pm$ SEM) | | Experimental 2(% $\pm$ SEM) | | Control 3(% $\pm$ SEM) | | Experimental 3(% $\pm$ SEM) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AI | F | AI | F | AI | F | AI | F | AI | F | AI | F |
| 3 h | 67.1 $\pm$ 13.8 | 65.6 $\pm$ 9.3 | 67.1 $\pm$ 13.8 | 65.6 $\pm$ 9.3 | 67.1 $\pm$ 13.8 | 65,6 $\pm$ 9.3 | 67.1 $\pm$ 13.8 | 65.6 $\pm$ 9.3 | 67.1 $\pm$ 13.8 | 65.6 $\pm$ 9.3 | 67.1 $\pm$ 13.8 | 65.6 $\pm$ 9.3 |
| 24 h | 48.5 $\pm$ 12.1 | 47.2 $\pm$ 1.9 | 55.1 $\pm$ 11.7 | 54.7 $\pm$ 8.3 | 50.5 $\pm$ 10.2 | 48.9 $\pm$ 1.7 | 56.2 $\pm$ 8.6 | 54.9 $\pm$ 2.4 | 49.3 $\pm$ 8.4 | 51.2 $\pm$ 3.2 | 55.1 $\pm$ 11.7 | 48.9 $\pm$ 1.7 |
| 48 h | 19.3 $\pm$ 10.1 | 17.8 $\pm$ 5.2 | 35.2 $\pm$ 6.8 | 36 $\pm$ 10.2 | 22.4 $\pm$ 11.5 | 22.1 $\pm$ 10.4 | 38.4 $\pm$ 8.5 | 36.6 $\pm$ 10.5 | 21.7 $\pm$ 12.2 | 21.6 $\pm$ 6.4 | 35.2 $\pm$ 6.8 | 22.1 $\pm$ 10.4 |
| 72 h | 12.6 $\pm$ 12.3 | 11.4 $\pm$ 11.6 | 24.2 $\pm$ 6.4 | 3.9 $\pm$ 8.5 | 15.6 $\pm$ 6.8 | 19.5 $\pm$ 9.6 | 28.4 $\pm$ 3.2 | 26.9 $\pm$ 10.2 | 20 $\pm$ 10.4 | 14.6 $\pm$ 6.8 | 24.2 $\pm$ 6.4 | 19.5 $\pm$ 9.6 |

Table 3
Capacitated and acrosome-reacted spermatozoa (Mean ± SEM) of fresh and cooled aliquots at different experimental times examined by CTC staining (B-AR) and by FITC-conjugated Pisum sativum staining (AR).

| | Control 1(% ± SEM) | | Control 2(% ± SEM) | | Control 3(% ± SEM) | | Experimental 1(% ± SEM) | | Experimental 2(% ± SEM) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AR | B-AR | AR | B-AR | AR | B-AR | AR | B-AR | AR | B-AR |
| 3 h | 8 ± 6.1 | 9 ± 4.3 | 8 ± 6.1 | 9 ± 4.3 | 8 ± 6.1 | 9 ± 4.3 | 8 ± 6.1 | 9 ± 4.3 | 8 ± 6.1 | 9 ± 4.3 |
| 24 h | 22.4 ± 4.1 | 23.2 ± 10.1 | 17.6 ± 6.2 | 18.1 ± 10.9 | 24.3 ± 7.3 | 25.2 ± 10.1 | 14.3 ± 2.3 | 16 ± 10.9 | 12.4 ± 4.2 | 13.1 ± 6.8 |
| 48 h | 49.16 ± 12.4 | 46.5 ± 3.4 | 40,16 ± 14.7 | 42.2 ± 5.3 | 42.16 ± 12.4 | 43.9 ± 7.6 | 37.6 ± 11.5 | 39.4 ± 5.7 | 34.1 ± 12.8 | 36.2 ± 9.1 |
| 72 h | 73.16 ± 11.2 | 69.8 ± 7.6 | 68.16 ± 10.4 | 69.8 ± 2.5 | 65.10 ± 3.4 | 62.4 ± 8.1 | 56.6 ± 3.8 | 58.4 ± 10.8 | 52.7 ± 2.5 | 50.9 ± 7.3 |

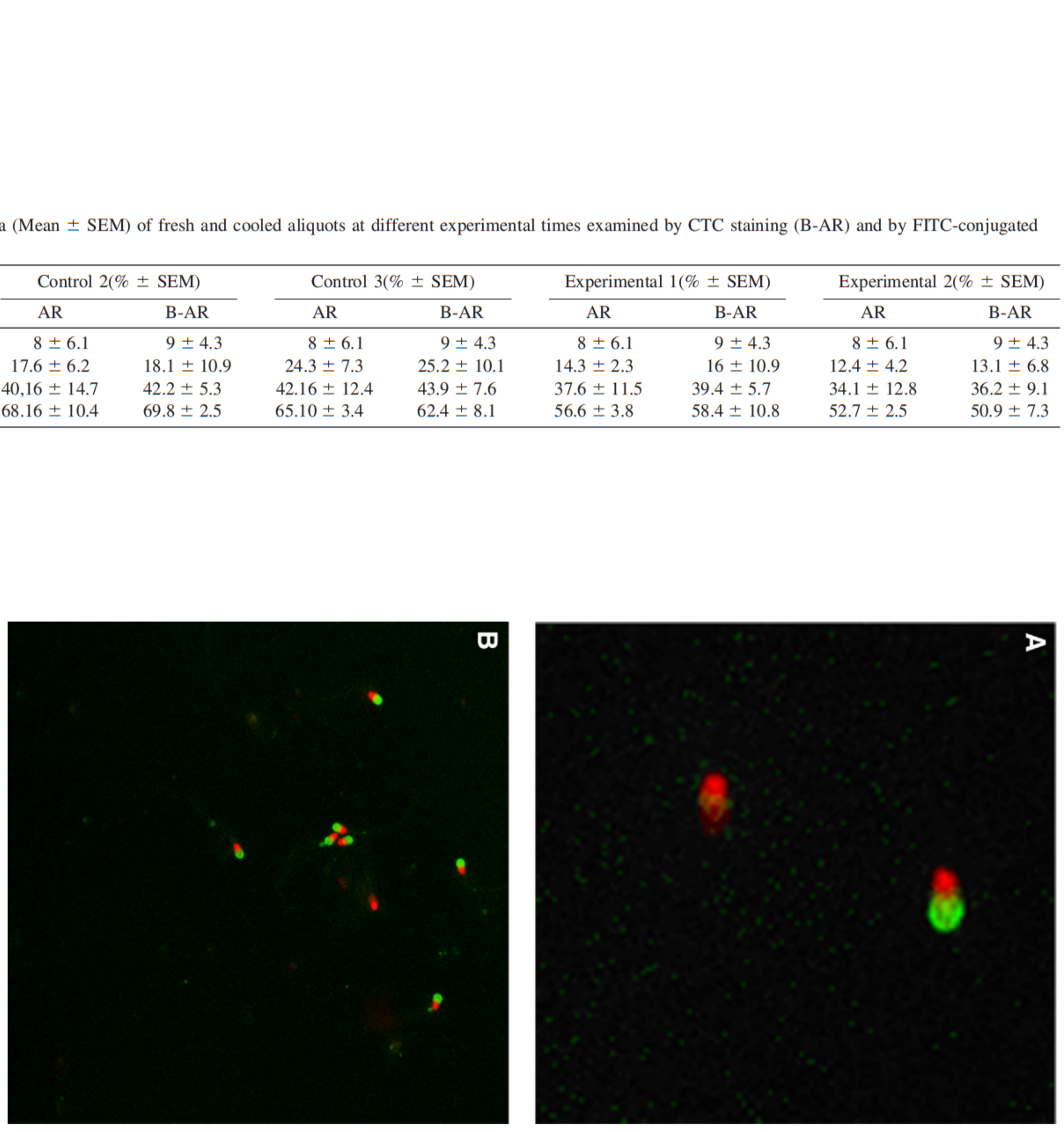


Fig. 2. Evaluation of acrosomal status by FITC-PNA staining methods. (a) Confocal microscope observation at 400 × magnification: spermatozoa with green fluorescent acrosome are classified as intact acrosome (AI); spermatozoa without this green fluorescent region are classified as acrosome reacted (AR). (b) Confocal microscopic observation at 100 × magnification, experimental 1 sample at 24h of storage.

24 h of storage, in native semen (control 1) and in semen diluted with Kenney semen extender (control 2) was 5,147 ± 7.2 and 4,214 ± 6.8 respectively; at time zero it was not possible to measure the optical density of p-ERK1 in semen treated with SOD (experiment 1 and 2).

Table 4
Western blotting evaluation of ERK (Extracellular signal-regulated kinase) protein phosphorylation quantified by densitometric analysis. Results were expressed as optical density.

| | 3h | | 24h | | 48h | | 72h | |
|---|---|---|---|---|---|---|---|---|
| | ERK1 | pERK1 | ERK1 | pERK1 | ERK1 | pERK1 | ERK1 | pERK1 |
| Native semen (Control 1) | 2896 ± 15.2 | 0 | 2984 ± 5.2 | 5147 ± 7.2$^{A}$ | 3184 ± 11.9 | 6147 ± 18.3$^{A}$ | 2978 ± 4.7 | 6256 ± 16.5$^{A}$ |
| Extended semen (Control 2) | 3100 ± 9.3 | 0 | 2947 ± 135 | 4214 ± 6.8$^{A}$ | 4105 ± 8.6 | 4650 ± 14.2$^{A}$ | 3911 ± 5.4 | 4926 ± 15.4$^{A}$ |
| Extended pellet (Control 3) | 2267 ± 12,8 | 0 | 2443 ± 12.9 | 4823 ± 18.2$^{A}$ | 3621 ± 5.9 | 5212 ± 15.6$^{A}$ | 3621 ± 5.2 | 5814 ± 18.2$^{A}$ |
| SOD 25UI (Experimental 1) | 2784 ± 11.7 | 0 | 4452 ± 15.6 | 0$^{B}$ | 4623 ± 11.6 | 1680 ± 13.6$^{B}$ | 4623 ± 5.6 | 2412 ± 11.9$^{B}$ |
| SOD 50UI (Experimental 2) | 2451 ± 5.8 | 0 | 3556 ± 13.6 | 0$^{B}$ | 3910 ± 12.7 | 2341 ± 9.2$^{B}$ | 3910 ± 15.8 | 2841 ± 12.3$^{B}$ |

$^{A–B}$ $P < 0.01$ (column).

At 48 and 72 h of storage, incubation with SOD was associated with a significant ($P < 0.01$) reduction of optical density of p-ERK1 compared with the control (Table 4). Statistical analysis showed a significant difference between control and experimental aliquots at 24, 48 and 72h of storage. These data showed that pERK1 level rise when sperm quality parameters undergo a significant reduction (Table 4).

## 4. Discussion and conclusion

In this study, we determined effects of antioxidative activity of a low concentration of SOD added to semen extender during storage at 5 °C on stallion sperm motility, viability and ERK phosphorylation. There are few studies on the effects of adding antioxidants to semen extenders during cooling and/or freezing of mammalian spermatozoa [11]. Recent studies show that there is a physiological SOD activity in human seminal plasma [14,24]. However, *in vitro* addition of a low dose of SOD (50IU/ml) was harmful to human sperm motility [27]. The SOD activity of human seminal plasma can be increased by oral administration of antioxidants [28]. Vitamin E and Glutathione (GSH) could protect boar semen against fatty acid peroxidation and there was a positive influence of vitamin E supplementation on semen quality; although the seminal plasma SOD and the oxidized form of GSH (Glutathione Disulfide–GSSG) tended to rise with the time of vitamin E administration, the increase did not reach a significant level by the seventh week [29].

Nevertheless, because SOD-like activity in equine seminal plasma is 29.2 ± 6.6 IU/mg of protein [18], we chose to use low doses of SOD in our experiments both to verify if in the horse the addition of SOD at a concentration equal to those tested on human semen have the same adverse effects on semen quality and in order to create similar conditions to those inducible with oral administration of antioxidants.

In stallion semen, ROS are generated mainly by damaged and abnormal spermatozoa and by contaminating leukocytes [18].

Antioxidant systems control the balance between production and neutralization of ROS and protect spermatozoa against peroxidative damage [13]. A previous study showed that lipid peroxidation may be a greater problem in frozen-thawed than in cooled semen. In this study, no increase in lipid peroxidation products during storage at 5 °C was found [11]. This indicates that lipid peroxidation apparently is not a major factor influencing semen viability during storage at 5 °C. These data confirm previous findings [19] that an artificially generated ROS excess decreased sperm motility but without increasing lipid peroxidation.

It was demonstrated that in equine semen stored at 5 °C the addition of antioxidant enzymes (GSH290 Px, SOD and CAT) increased sperm quality, but TBARS (Thiobarbituric acid reactive substances) content analysis

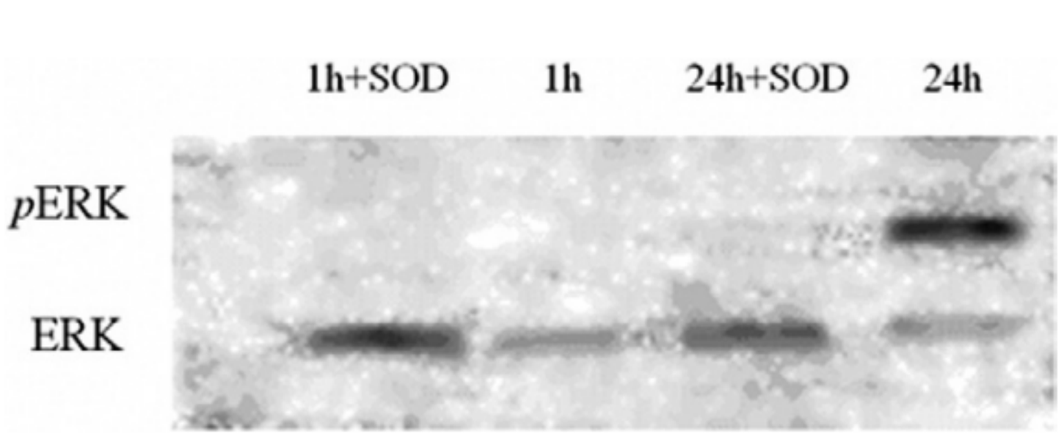


Fig. 3. Western blot gel of ERK (Extracellular signal-regulated kinase) protein and phosphorylated-ERK at different times (1h and 24 h) of storage in control 3 aliquots and in Experimental 1 aliquots (+ SOD).

revealed no increase in the lipid peroxidation products when equine spermatozoa were cooled in extender without the antioxidant enzymes [11]. These data suggest that antioxidant defence systems protect spermatozoa efficiently and thus lipid peroxidative ROS damage is not the major reason for the decline in motility, so perhaps different pathogenetic mechanism are involved.

Another study shows that stallion sperm DNA stored for 31 h at 5 °C did not show changes in chromatin structure [30].

It was suggested that peroxidative stress triggers the MAPKs cascade and ERK phosphorylation [12]. Phosphorylation of proteins plays an important role in capacitation, the acrosome reaction and sperm penetration of the zona pellucida. Nevertheless, almost nothing is known regarding the nature of the endogenous and physiologically relevant sperm-ligands able to phosphorylate and activate the various MAPKs (ERK, JNK and p38) [12]. The MAPKs ERK1/2, and their cascade members Sos/Raf-1/MEK1, were identified in the tail of mature ejaculated human spermatozoa by immunocytochemistry [12]. The presence of active ERK1/2 was evaluated by Western blotting and ERK1/2 was reported to be a parameter of poor, stressed spermatozoa [12].

In this study, effects of semen extenders with or without different concentrations of SOD on quality of stallion spermatozoa stored at 5 °C were determined. All quality parameters analyzed declined progressively during cooling in all samples. However, semen in Experiment 1 and Experiment 2 aliquots, treated with SOD, showed higher quality parameters (motility, viability and AI) compared to native semen and to spermatozoa diluted with simple extender at 24, 48 and 72 h of storage. The hypothesis that elevated ROS occurs over time and that SOD addition to semen extender will have a positive effect on the fertility potential of spermatozoa was tested in this study by assaying the ERK pathway during 5 °C storage. Interestingly, total pERK1 was inversely correlated with the sperm quality parameters. After 24 h the semen samples that did not contain antioxidants demonstrated a significant decrease of the quality parameters and a significant increase in the amount of active pERK1 proteins. Our result confirms the hypothesis of a relationship between increasing ROS, reduction of sperm motility and vitality and increasing of ERK protein phosphorylation during equine semen storage at 5 °C. The addition of SOD at the low concentration tested increased the sperm quality parameters during 72 h of storage at 5 °C and reduced the ERK protein phosphorylation. The addition of SOD to the semen extender could prolong storage of stallion semen, allowing longer distance shipments and a more precise timing of insemination thereby further increasing the already high rates of fertility. Moreover, antioxidant addition might also further benefit spermatozoa within the female reproductive tract [31]. During post-breeding endometritis, large numbers of neutrophils enter the uterus and release a number of chemical mediators, including oxygen-free radicals. Prolonged exposure to oxygen-free radicals, as seen in mares with delayed uterine clearance, increases the likelihood of cell damage [31]. It is possible that the addition of antioxidants to the sperm extender could reduce the intra-uterine production of ROS.

In conclusion, our findings support the hypothesis that in stallions ROS are responsible for the deterioration in quality of semen stored at 5 °C, and showed that the addition of SOD to the semen extender improves the quality of cold-stored stallion semen. Our data allow us to hypothesize that the underlying pathogenic mechanism of damage to equine sperm cells by ROS is the alteration of ERK proteins, and The ERK levels can be used as a marker/indicator of sperm damage. Fertility of cooled stallion semen is maintained for 24–48 h. After that time, pregnancy rates decrease dramatically [1]. Our results offer additional information to the already large international database for the development of new extenders designed to ensure a longer storage period. Further studies are needed to evaluate the antioxidant enzyme activities in seminal plasma, and the generation of ROS during semen cooling, in order to verify if antioxidant supplementation will improve in vivo equine fertility rates of chilled semen, to find other appropriate antioxidants and to define the most effective concentrations. In the meantime, we are comparing the effects of a new isoform of rMnSOD (Manganese Superoxide Dismutase recombinant in *E. coli*) isolated by human liposarcoma that is currently used in cancer therapy research [32], together with the commercial SOD employed in this study. This new isoform of rMnSOD is able to penetrate into the cytoplasm of cells that produce estrogen receptors. Its penetration into sperm cells must be demonstrated but the preliminary results obtained indicate that lower concentrations of the new isoform of rMnSOD are more effective at raising semen quality parameters than presently-available antioxidant enzymes.

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