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Addition of Serine protease inhibitors on equine sperm extender during cooling and freezing

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Summary

The objective of the present work was to examine the consequences of adding serine protease inhibitor and its synthetic analogs to stallion semen during cryopreservation in order to reduce cryocapacitation during cooling and freezing. Semen from six stallions was collected, and samples were cooled to 5°C following the addition of serine proteases inhibitors a) Benzamidine (5mM, 7.5mM, and 9mM); b) Berenil (1mM, 2.5mM, and 5mM); c) purified inhibitor (4mM) in final concentrations, and other samples were frozen with INRA 82 freezing extender in liquid nitrogen, following the addition of serine proteases inhibitors. Sperm progressive motility was evaluated under phase contrast microscopy, and membrane functional integrity with the hyposmotic swelling test (HOST). Sperm membrane integrity was assessed with propidium iodide stain and acrosome integrity (AI) with FITC-PNA stain, using a flow cytometer, and induction of acrosome reaction with calcium ionophore A23187 (5μM). In the control and treated groups, respectively, the mean values of progressive motility (47.5 % and 48.7 %) and membrane integrity (68.0% and 63.8%) after cooling and of progressive motility (10.8% and 11.8%), membrane integrity (21.4% and 23.4%), HOST+ (24.7% and 25.9%) and acrosome reaction (3.8% and 3.4%) after thawing, did not differ ($p>0.05$). After the induction of acrosome reaction, no difference ($p>0.05$) was observed in acrosome reacted sperm between the control (12.7%) and treated groups (16.8%). The tested serine protease inhibitors could not reduce stallion sperm membrane scrambling which occurs during cooling and freezing.

Keywords: sperm, stallion, semen, cryopreservation, serine protease inhibitor

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Zusatz von Serin-Protease-Inhibitoren zur Konservierung und Verdünnung des Hengstsamens während Abkühlung und Tiefgefrierung

Während der Samenkryokonservierung stellen sich oftmals Membranschäden in Samenzellen ein, wie zum Beispiel Samenzell-Kapazitation oder Kryokapazitation. Der Zusatz von Proteinen zu Hengstsamen kann die Überlebensrate der aufgetauten Spermien und somit auch die Fertilisierungskapazität verbessern. Proteine wie die Serin-Protease-Inhibitoren kontrollieren den Kalziumeintritt in die Spermienzellen und die Akrosomreaktion durch Hemmung der Akrosin-Aktivität während der Spermienkapazitation. Bisher wurde jedoch noch nicht untersucht, ob der Zusatz von Serin-Protease-Inhibitoren und deren synthetische Analoge die Kryokapazitation während der Kryokonservierung von Hengstsamenzellen verringern kann. Das Ziel der vorliegenden Arbeit war die Kryokapazitation von Henstspermien zu untersuchen und durch Zugabe von Serin-Protease-Inhibitoren und deren synthetischer Analoge zu Samenproben während der Abkühlungs- und Tiefgefrierungsphase die Samenqualität zu verbessern. Insgesamt wurden Samenproben von 6 Hengsten aufgenommen und nach Zugabe von Serin-Protease-Inhibitoren, a) Benzamidine (5mM, 7,5mM, und 9mM); b) Berenil (1mM, 2,5mM, und 5mM); c) natürlicher Inhibitor (4mM der Endkonzentration), auf bis zu 5°C abgekühlt. Weitere Samenproben derselben Hengste wurden mit Serin-Protease-Inhibitoren versetzt und mit INRA 82 Gefriermedium in Stickstoff tiefgefroren. Die Motilität der Samenproben wurde mit Hilfe eines Phasenkontrastmikroskopes auf einem auf 38°C erwärmten Heiztisch bei 200-facher Vergrößerung im Hellfeldverfahren beurteilt. Die prozentualen Anteile der vorwärts-, orts- und unbeweglichen Spermienzellen wurde ermittelt. Um die funktionelle Integrität der Spermienmembran zu untersuchen wurde der hypoosmotische Schwellungstest (HOST) verwendet. Dieser wurde mit Hilfe eines Phasenkontrastmikroskopes und einem auf 38°C erwärmten Heiztisch bei 400-facher Vergrößerung im Hellfeldverfahren durchgeführt und beurteilt. Der Anteil auf den osmotischen Stress positiv reagierender Spermien (Prozentsatz an geschwollenen Spermien) wurde als funktionelle Spermienmembran angesehen. Zur Bewertung der Membranintegrität und der Akrosomintegrität von Samenzellen wurden die Fluoreszenzfarbstoffe Propidiumiodid und die FITC-PNA Färbung eingesetzt und die Samenzellen mittels Durchflusszytometrie untersucht. Die Akrosomenreaktion der Hengstspermien wurde mit dem Kalzium-Ionophor A23187 (5μM) induziert und ebenfalls mittels Durchflusszytometrie untersucht. Kontrollgruppe und behandelte Gruppe zeigten einen prozentualen Anteil der Samenzellen mit Vorwärtsmotilität von 47,5 % bzw. 48,7 % und eine Membranintegrität von 68,0% bzw. 63,8% nach Abkühlung der Samenproben. Nach dem Auftauen wurde in Kontroll- und behandelter Gruppe eine Vorwärtsmotilität von 10,8% bzw. 11,8%, eine Membranintegrität von 21,4% bzw. 23,4%, HOST+ von 24,7% bzw. 25,9% und Akrosomreaktion von 3,8% bzw. 3,4% ermittelt ($p>0,05$). Die in der vorliegenden Arbeit benutzten Serin-Protease-Inhibitoren konnten die während der Abkühlung und Tiefgefrierung vorkommenden Membranschädigungen in den Pferdespermien nicht vermindern. Nach dem Einsetzen der Akrosomreaktion wurde kein signifikanter Unterschied hinsichtlich dem Anteil von Akrosom-reagierenden Spermien ($p>0,05$) zwischen Kontrolle (12,7%) und behandelter Gruppe (16,8%) festgestellt. Seminalplasma aus Ejakulat enthält Serin-Protease-Inhibitoren die, einmal verbunden mit der Serin-Protease, die Wirkung auch von anderen Inhibitoren unterbinden. Dies trifft hauptsächlich für frisches Ejakulat zu, bei dem die Stöchiometrie der Reaktion 1:1 beträgt. Obwohl der Anteil von Akrosom-reagierenden Spermien in beiden Gruppen ähnlich war, könnte es dennoch vorteilhaft sein, die Inhibitoren zu einem früheren Zeitpunkt während der Konservierung des Samens einzusetzen. Der Zusatz von Serin-Protease-Inhibitoren zum Samen unmittelbar nach Entnahme könnte die Verdünnungswirkung der Samenverdünner vermindern. Au erdem könnte während der Samenabkühlung und Tiefgefrierung die Spermienmembran beschädigt werden und infolgedessen eine Inaktivierung der Inhibitoren eintreten. Der Zusatz von Serin-Protease-Inhibitoren zum Kühlmedium während Abkühlung und Tiefgefrierung von Samenproben konnte die Spermien-Kryokapazitation nicht vermindern. Ein positiver Effekt von Serin-Protease-Inhibitoren auf die Kapazitation der Hengstsamenzellen und auf die Qualität von kryokonservierten Samenzellen konnte somit nicht nachgewiesen werden.

Schlüsselwörter: Spermien, Pferd, Samen, Kryokonservierung, Serin-Protease-Inhibitoren

Introduction

Although there are several studies on horse semen cryopreservation (*Cochran* et al. 1984, *Dobrinsky* et al. 1995, *Ball* and *Vo* 2001, *Pommer* et al. 2003, *Sieme* et al. 2004, *Vidament* 2005, *Alvarenga* et al. 2005, *Moore* et al. 2005) there is not yet a method to preserve the semen of most stallions. During cryopreservation, damage such as sperm capacitation or "cryocapacitation" often occurs to the sperm plasma membrane (*Ashworth* et al. 1994, *Watson* 2000, *Pommer* et al. 2003). The addition of proteins to horse semen in order to reduce impaired fertilization could improve post-thaw sperm survival. Proteins, such as serine protease inhibitors, deactivate adenilate ciclase and control Ca^{2+} influx and acrosome reaction by inhibiting acrosin activity (*Okamura* et al. 1990). The functions of serine protease inhibitors on sperm capacitation and acrosome reaction have been studied (*Green* 1978, *Lui e Meizel* 1979, *Fraser* 1982); however, no studies have examined their effect on preventing cryocapacitation as a result of sperm cryopreservation. The objetive of the present work was to test whether the addition of serine protease inhibitor and its synthetic analogs to stallion semen during cryopreservation reduces "cryocapacitation" during cooling and freezing.

Material and Methods

Chemicals

Propidium iodide (PI) and Carboxifluorescein diacetate (CFDA) stains and Ca^{2+} ionophore A23187 were purchased from Sigma Chemical (St. Louis, MO, USA) and FITC-PNA stain was a product of EY Laboratories Inc. (San Mateo, CA, USA).

Semen collection

Semen from six stallions (4 to 12 years old) was collected with an artificial vagina. Sperm progressive motility (100x) and morphology (1000x) were assessed using a phase contrast microscope (*Mies Filho* 1975), and sperm cell number/mL was determined using a haemocytometer chamber. Only semen with a minimum 50% progressive motile sperm was used. The ejaculates were divided into samples according to cooling and freezing procedures described as follows.

Cooled semen

Semen was resuspended with Kenney extender (*Kenney* et al. 1975) to a sperm concentration of $25x10^6$ spermatozoa/mL. A semen sample of 1 mL was considered the control and the other 1 mL semen samples were added to tubes containing different amounts of serine proteases inhibitors: a) 5 ml Benzamidine, b) 7.5 ml Benzamidine, c) 9 ml Benzamidine, d) 1ml Berenil , e) 2.5 ml Berenil , f) 5ml Berenil, g) 4 ml purified inhibitor. The end concentration of the natural serine protease inhibitor was equal to that found in equine seminal plasma; however, due to fluctuating inhibitory activity, its synthetic analogs Benzamidine (5mM /$100x10^6$ spermatozoa) (*Fraser* et al. 1982) and *Berenil* (1mM /$100x10^6$ spermatozoa) were used in varying concentrations. Berenil showed an inhibitory activity ten times higher than Benzamidine. The purified inhibitor was obtained as described by *Vasconcelos* et al. (2009). Semen samples were added to tubes and cooled to 5 °C over a two-hour period (0.16° C/min) and maintained at 5°C for another two hours. Four hours thereafter the semen was evaluated.

Frozen semen

Semen samples were diluted with Kenney extender (1:1) and centrifuged (500xg, 10 min). The supernatant was discarded, the sperm pellet homogenized, and sperm number/mL calculated. Semen was resuspended with INRA 82 extender (*Vidament* et al. 2001) until a sperm count of $100x10^6$/mL was achieved. A semen sample of 1 mL was considered as the control, and the other 1 mL semen samples were added to tubes containing different amounts of serine proteases inhibitors: a) 5 ml Benzamidine, b) 7.5 ml Benzamidine, c) 9 mL Benzamidine, d) 1ml Berenil , e) 2.5 ml Berenil , f) 5ml Berenil, g) 4 mL purified inhibitor. Semen samples were packaged in 0.5 mL straws and cooled from 36 °C to 24.1 °C (1.19 °C/min) and from 24.1 °C to 4.9 °C (0.48 °C/min) over a 50-minute period. Next, the straws were frozen in liquid nitrogen vapor for 20 minutes and then plunged in liquid nitrogen. Straws were thawed at 75 °C for 7 seconds and then at 37 °C for 30 seconds. After thawing, the samples were evaluated.

Evaluation of cooled semen

The percentage of sperm, in cooled semen, with progressive motility was determined under phase contrast microscopy (100x). Sperm with intact membrane were identified using Propidium iodide and Carboxifluorescein diacetate stains (FITC-CFDA/PI) (*Harrison* and *Vickers* 1990) under epifluorescent microscopy (1000x Olympus BX41, wave length 620nm, emission 488/515 nm). After examining 200 sperm cells, only those that were 100% fluorescent green were considered membrane intact.

Evaluation of frozen semen

The percentage of progressive motile sperm of frozen semen was determined using phase contrast microscopy (100x). To assess membrane functional integrity of the sperm, a modified hypo-osmotic swelling test (HOST) (*Lomeo* and *Giambersio* 1991) was used. This was carried out by adding 100 µL of semen to 200 µL of distilled water (*Lagares* et al. 1998) at 37 °C for 5 min. The swelling reaction of the sperm cells after hypo-osmotic stress was measured by phase contrast microscopy (400x). After examining 200 cells, the number of positive reacted sperm (tail swelling) was calculated and subtracted from the number of sperm with tail swelling before the osmotic stress. Sperm membrane integrity was evaluated after diluting samples 1:20 in phosphated buffer solution (PBS) (250 µL semen in 5 mL PBS) at 37°C. A sample of 200µL was added to tubes containing 30 µL propidium iodide (1.5 mM) and 1 mL PBS, which were kept at room temperature in the dark for 10 min and then assessed with a flow cytometer (FACScan, Becton Dickinson®, San Jose, CA, USA)(Figure 1). Acrosomal integrity was evaluated by adding 15µL of lectin Peanut agglutinin (FITC-PNA) to 200µL of the propidium iodi-

de diluted sample (final conc. 1.125 µg/mL) and incubated for 10 min at 37 °C (Figure 1).

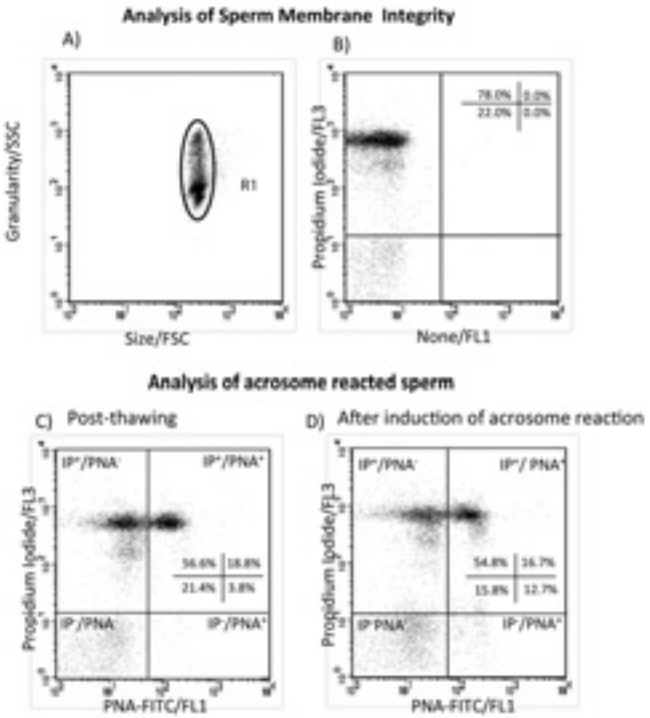

Fig. 1 Evaluation of stallion sperm by flow cytometry. **A** Sperm cells were selected based on their size and granularity laser scatter properties. **B** Sperm membrane integrity was assessed with PI stain and classified as intact membrane (PI-) or damaged membrane (PI+), **C** post-thawing evaluation of acrosome reaction and **D** after in vitro induced acrosome reaction. PI+/PNA-: Sperm cells with non-intact plasma membrane/intact acrosome, PI+/ PNA+: Sperm with non-intact plasma membrane and acrosome; PI-/PNA-: Sperm with intact plasma membrane and acrosome and PI-/PNA+: Sperm with intact plasma membrane/non-intact acrosome.
*Beurteilung von Hengstsamenzellen mittels Durchflusszytometrie. **A** Die Spermien wurden entsprechend ihrer Größe und Granulosität im Laser gemessen. **B** Spermien mit intakter Membran (PI-) oder nicht intakter Membran (PI+) wurden mit PI-Färbung beurteilt. Beurteilung des Spermienanteils mit Akrosomreaktion nach Auftauen **C** und nach Induzierung der Akrosomreaktion **D** tiefgefrorener Samenproben. PI+/PNA-: Spermien mit nicht intakter Membran/intaktem Akrosom; PI+/ PNA+: Spermien mit nicht intakter Membran und Akrosom; PI-/PNA-: Spermien mit intakter Membran und Akrosom; PI-/PNA+: Spermien mit intakter Membran/nicht intaktem Akrosom.*

Acrosome reaction rate of frozen-thawed semen after induction

In order to determine the "in vitro" fertilization capability of equine sperm after freezing-thawing, acrosome reaction was induced with calcium ionophore A23187 as described by *Landim-Alvarenga* et al. (2004). A stock solution of (0.95mM) of calcium ionophore was prepared. From that solution, 5µL were added to 0.5mL of semen containing 50x10^6 sperm. After a 30 min incubation time at 37 °C, sperm membrane integrity and the rate of acrosomal reaction were evaluated with FITC-PNA/PI using a flow cytometer (FL3 photodetector).The samples were analyzed using a FACScan flow cytometer. After transmitting a 560-nm short-pass dichroic mirror, the green fluorescence (FL1) was collected through a 515- to 545-nm band-pass filter. The red fluorescence (FL3) was collected through a 650-nm long-pass filter, after a 640-nm long-pass filter. The sheath/ sample was set on ''low'' and adjusted to a flow rate of 200 cells per second when analyzing a sample with a concentration of 1.25 x10^5 sperm/mL. Data acquisition of 10,000 cells was collected in list mode using BD CellQuest Pro version 4.0 software (Becton Dickinson®, San Jose, CA, USA). Analysis flow cytometric plots of propidium iodide/forward-scatter was done with double dye PI/PNA-FITC (Figure 2).

Statistical Analysis

Fisher's and Student-Newman-Keuls tests were used to analyze progressive sperm motility, sperm with a physical and functional membrane, acrosome integrity, and rate of induced sperm acrosome reaction after cooling and thawing, expressed as means and standard deviation (SD). The complete data was analyzed using the SAS program (Statistical Analysis System). A probability of $P<0.05$ was considered significant.

Results

There was no statistic difference ($P> 0.05$) in the percentage of progressively motile sperm and intact sperm plasma membranes of cooled semen between the control group and that treated with serine protease inhibitors (Figure 2).

Post-thaw sperm progressive motility (10.8% and 11.8%), membrane integrity (21.4% and 23.4%), functional sperm membrane (24.7% and 25.9%), and spontaneous acrosome reacted sperm (3.8% and 3.4%) did not differ between the control and the treated groups, respectively ($P>0.05$) (Figure 2). After induction, there was no difference between the rate of acrosome reacted sperm in the control group and the treated groups, suggesting that the serine protease inhibitors tested could not impair sperm capacitation and acrosome reaction (Figure 2).

Discussion

In the present work, serine protease inhibitors were added to stallion semen before cooling and freezing, respectively, in order to impair sperm capacitation and acrosome reaction as a result of cryocapacitation. Nevertheless, this effect was not observed in the present work, because there was no difference found among the seminal characteristics after addition of serine protease inhibitors to equine semen. During sperm capacitation, the adenilate ciclase promotes AMPc production, which in turn activates protein kinase A (PKA), inducing tirosine phosphorylation and the formation of substrates. These substrates in the presence of AMPc promotes ATP production, which is responsable for sperm motility (*Salem* et al.

1992). In mice (*Lui* and *Meizel* 1979, *Fraser* 1982), Guinea pig (*Perreault* et al. 1982) and pigs (*Winnica* et al. 2000) it has been suggested that serine protease inhibitors, such as p-Aminobenzamidine and Benzamidine, could inactivate the centage of motile sperm, intact membranes, nor acrosome reacted sperm after induction, suggesting that the addition of these inhibitors to cryopreservation medium was not beneficial in protecting equine sperm from cryocapacitation.

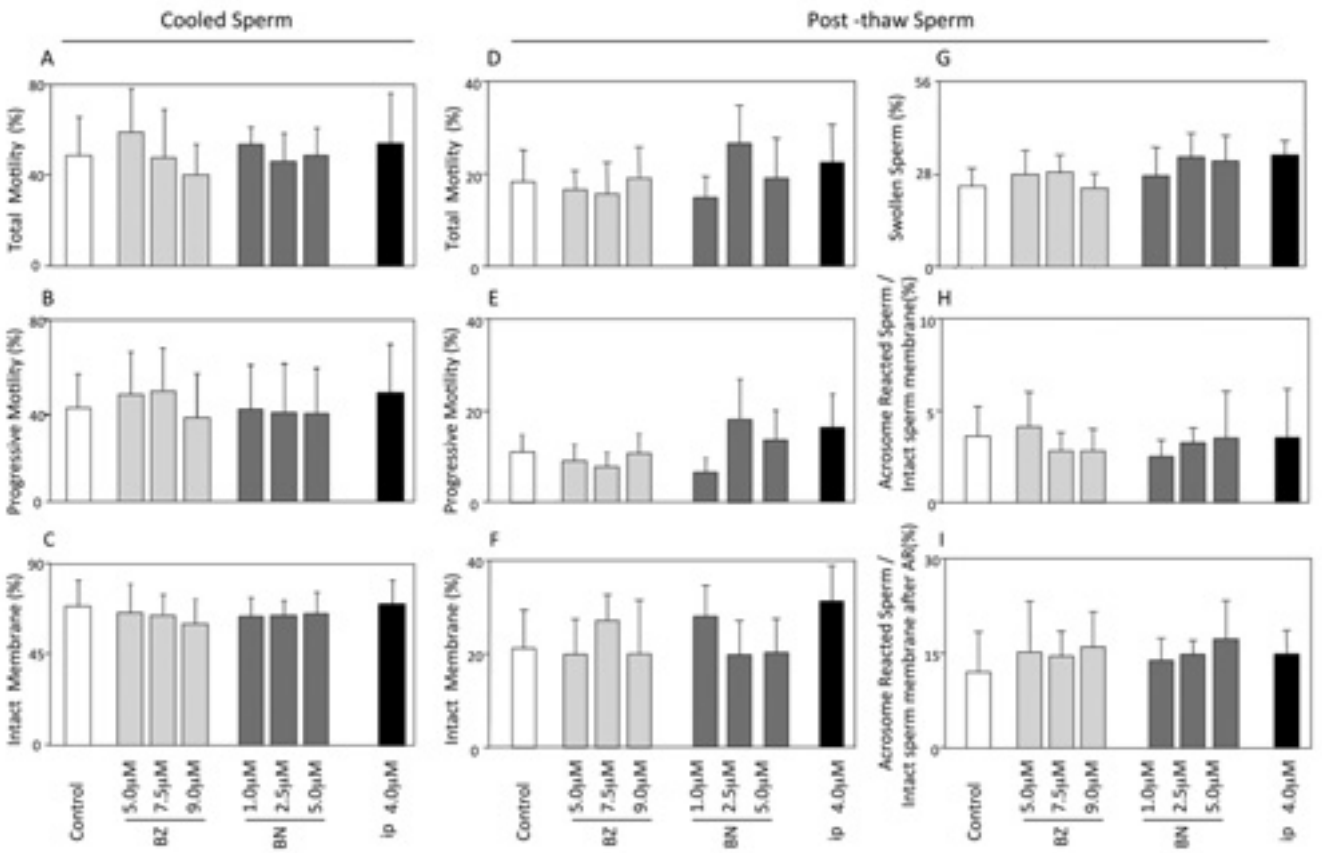

Fig. 2 Evaluation of cooled and frozen-thawed stallion sperm. Mean ± SD. Percentage of total sperm motility (**A** and **D**), progressive sperm motility (**B** and **E**) and intact sperm membrane (PI stain) (**C** and **F**) evaluated in cooled and frozen-thawed semen, respectively. Percentage of swollen sperm (HOST) (**G**) and sperm with an intact plasma membrane with a spontaneous (**E**) and induced acrosome reaction (**F**), respectively. Control: semen frozen with INRA 82 extender, BZ: Benzamidine, BN: Berenil, ip: Purified serine protease inhibitor (P>0.05).
*Beurteilung von abgekühlten und tiefgefrorenen Hengstsamenzellen. Mittelwerte ± SD. Anteil der Gesamt- (**A** und **D**) und der Vorwärtsmotilität (**B** und **E**) von Spermien und Anteil von Spermien mit intakter Membran (PI-Färbung) (**C** und **F**), jeweils nach Abkühlung und Auftauen. Prozentsatz an geschwollenen Spermien (HOST) (**G**) und Spermien mit intakter Membran und spontaner Akrosomreaktion (**E**) und nach induzierter Akrosomreaktion (**F**). Kontrolle: Samen wurde tiefgefroren mit INRA 82 Verdünner, BZ: Benzamidine, BN: Berenil, ip: Aufgereinigte Serine- Protease-Inhibitoren (P>0,05).*

adenilate ciclase from epididimal sperm of swine reducing sperm motility (*Okamura* et al. 1990), and reducing acrosome reaction in the mouse (*Fraser* 1982, *Lai* 1982). In the ejaculated semen, the seminal plasma contains serine protease inhibitors, which are bound to the serine proteases preventing the action of others inhibitors. This occurs mainly in fresh semen where the stochiometry of the reaction is 1:1 (*Ball* et al. 1997, *Jonakova* et al. 2000). Since the rate of induced acrosome reaction observed in stallion sperm in the present work was similar in all groups, it may have been advantageous to add the serine protease inhibitors at an earlier step in semen processing, possibly immediately after semen collection. This may have reduced the dilution effect of the extender.

Also, damage to sperm membranes due to the semen cooling and freezing process could have inactivated inhibitors. This is the first time that serine protease inhibitors were added to semen during cryopreservation in order to impair equine sperm cryocapacitation. However, it did not improve the per-

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