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Assessment of Fertilizing Ability of Frozen-thawed Stallion Semen in Glutamine Extender

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Abstract: The fertilizing ability of stallion spermatozoa frozen-thawed in glutamine extender was evaluated, using servel functional tests: post-thaw motility, chromatin structure, plasma membrane integrity, acrosomal status, and sperm-zona pellucida binding. Fifteen ejaculates were collected from 3 fertile stallions. Each ejaculate was divided into 2 parts, respectively frozen in INRA freezing extender (Control: INRA82 + 2% (v/v) egg yolk + 2.5% (v/v) glycerol + 20mM hepes) and Glutamine (control + 50mM glutamine) extenders. Semen functional characteristics were evaluated after thawing, by computer automated sperm analyser, acridine orange (AO) staining, fluorescein isothiocyanate-Pisum sativum agglutinin (FITC-PSA) technique, hypo-osmotic swelling (HOS) test, and hemi zona assay (HZA). Results showed that glutamine extender afforded more protection to stallion spermatozoa than control extender. It was concluded that glutamine at 50mM in INRA freezing extender could enhance fertility of stallion semen after freezing and thawing.

Key words: Stallion, spermatozoa, functional tests, fertility, glutamine

Introduction

Artificial insemination with cooled and frozen semen has allowed an increase in the use of semen from breeder stallions of high genetic interest. However, frozen semen has more limited use than fresh and cooled semen for a number of reasons. The principal reason is the low fertility of frozen-thawed semen though it's contained an adequate number of progressively motile sperm (Amann and Pickett, 1987). This reduction in fertility is partially caused by the freeze-thaw process (Hammerstedt *et al.*, 1990) and the use of toxic conventional cryoprotectants such as glycerol in the freezing medium (Demick *et al.*, 1976; Fahy, 1986; Fahy *et al.*, 1990 and Jasko *et al.*, 1992). Thus, the necessity to predict fertilizing ability of frozen semen is important, in order to select which straws could be used for artificial insemination, with acceptable fertility rates.

The prediction of stallion fertility in vitro is often based on assessment of semen characteristics, by conventional methods such as number, concentration, motility and morphology. However, data on the relationship between sperm parameters and fertility are still conflicting (Dowestt *et al.*, 1982; Voss *et al.*, 1981; Jasko *et al.*, 1992 and Chevalier-clement *et al.*, 1991). Recently, current methods based on the evaluation of functional characteristics of spermatozoa, such as sperm viability estimated by evaluation of chromatin structure (Kenney *et al.*, 1995), plasma membrane integrity (Casey *et al.*, 1993 and Papaioannou *et al.*, 1997), acrosomal status (Farlin *et al.*, 1992 and Meyers *et al.*, 1995), and Hemi Zona Assay (Fazeli, 1995) were developed for assessing stallion semen quality.

It has been demonstrated that supplementation of freezing extender with glutamine improved post-thaw motility of human (Renard *et al.*, 1996), jackass (Trimeche *et al.*, 1996), and stallion (Trimeche *et al.*, 1999) semen. The objective of this study is to evaluate the effect of glutamine in preserving stallion fertilizing ability, using several functional tests: acridine orange (AO), staining fluorescein isothiocyanate-pisum sativum agglutinin (FITC-PSA), the hypo-osmotic (HOS) test, and hemi zona assay (HZA).

Materials and Methods

Semen Collection and Freezing: Semen was collected three times per week from 3 stallions, using a Missouri-model artificial vagina. Five ejaculates from each stallion were used (N = 15). Immediately following collection, the gel-free portion of the ejaculate was evaluated for volume, sperm concentration, and percentage of progressively motile sperm. Semen was frozen according to Vidament *et al.* (1997). Two cyropreservation extenders were used in this study. The INRA freezing extender (Control) was composed of modified INRA 82 medium (1 L saline solution: 50 g glucose, 3 g lactose, 3 g raffinose, 0,49 g sodium citrate dihydrate, 0,82 g potassium citrate, 9.52 g Hepes, 100 000 IU penicillin, 100 mg gentamycin and 1 L skim milk) supplemented with 2% (v/v) egg yolk and 2.5% (v/v) glycerol (Vidament *et al.*, 1997). The Glutamine extender was composed of control extender supplemented with 50mM L-glutamine. Each ejaculate was divided into 2 parts, respectively frozen in Control and Glutamine extenders.

Motility Assessment: Sperm motility after thawing was analysed with the ATS-20 automated analyser (J.C. Diffusion International, la Ferté-Fresnel, France), using

a 10μm depth Makler counting chamber. For each ejaculate, 3 straws of each freezing extender were thawed in a 37 °C water bath for 30 seconds.

To assess the nucleus integrity, acrosomal status, plasma membrane integrity, after freezing, sperm was diluted in a modified Hank's salt medium supplemented by 20mM Hepes and 1% of Bovine Serum Albumine (HHBSA) (Magistrini *et al.*, 1992) to a final concentration of 20 X 10^6 spermatozoa /ml. The Hank's salt medium was composed of 0.14 g $CaCl_2$, 0.4 g KCl, 0.06 g KH_2PO_4, 0.2 $MgSO_4 7H_2O$, 8 g NaCl, 0.118 g Na_2HPO_4, 12 H_2O, 0.35 g $NaHCO_3$, 1g glucose $C_6H_{12}O_6$, and distilled water for a final volume of 1000 ml.

Nucleus Integrity Assessment: This method has been described by Ballachy *et al.* (1988). For measurement, 20 μl of semen suspension was diluted with 180 μl of TNE buffer (0.01 M Tris-HCL, 0.15 M NaCl and 1mM disodium EDTA, pH 7.4) to give a concentration of approximately 1 to 2 10^6 spermatozoa per ml. This was mixed with 400 μl of acid-detergent solution (0,1% (v/v) Triton X-100, 0,08 NHCl, et 0,15 M NaCl, pH 1,2). After 30 seconds, 1.2 ml of an acridine orange staining solution (0.2 M Na_2HPO_4, 1mM disodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate, with 6μg acridine orange/ml, pH 6.0) was added. Then, after 2 to 3 minutes of incubation, air-dried preparations were made by smearing 0.8 μl of sperm suspension over the end of a pre-cleaned slide. They were then rinsed with de-ionised water and a coverslip placed befor the slide could dry. The coverslip was sealed with nail polish.

Slides were read the same day on a fluorescent microscope using a 490-nm excitation filter and a reflected light illuminator for fluorescence (Ploemopar 2.1). Normal sperm heads showed a green tinge, which is quite characteristic and discernible from yellow to red-colored cells, which are considered abnormal. A total of 100 spermatozoa were screened for each sample.

Acrosomal Status Assessment: This technique was adapted from that described by Meyers *et al.* (1995), Cazy *et al.* (1993) with stallion sperm. A 100 μl aliquot of diluted sperm was fixed with 100 μl of 2% (w/v) Paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS), pH 7.4. After 8 minutes of incubation, 5 μl of sperm suspension was collected on a polycarbonate membrane filter (2 μm pore-size, Whatman International Lid, Maidston, England) held in a disc filter holder. The filter disc was fitted with a vacutainer tube as diagrammed by Morales and Cross (1989) that was connected to an evacuation system by negative pressure. The sperm were washed by drawing 2 ml of DPBS over the filter with negative pressure created by system of evacuation. Absolute ethanol (200 μl previously filtrated (0.45 μm pore size) were added to permeabilize the sperm membranes. After 10 minutes, the filters were rinsed with 2 ml of DPBS to remove the ethanol then 200 μl of blocking solution (5% (w/v) BSA, 1mg/ml Sodium Azide in PBS (phosphate-buffered saline) were added to the filters. After 10 minutes of incubation, the blocking solution was drawn through the filter and 100 μl of 100 μg/ml FITC-PSA in DPBS was added. After a further incubation period of 10 minutes, the filters were rinsed with a small volume of blocking solution followed by 2 ml DPBS. Then, the filter was mounted on a microscope slide with 3.5 μl of 100 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO ; (Sigma Chemical Co, st Louis, MO) in absolute ethanol. DABCO inhibits photobleaching by excitation of fluorophores during microscopic observation. Then, nail polish was applied to the edges of the coverslip before the slide could dry. Slides were read the same day on a fluorescent microscope using a 490-nm excitation filter and a reflected light illuminator for fluorescence (Ploemopar 2.1). The acrosomal status of 100 spermatozoa was assessed. Spermatozoa lacking green fluorescence over the acrosomal region were discernible from other stained cells and classified as acrosome intact. Those spermatozoa that displayed a distinct homogeneous green fluorescence over the acrosomal region or thin band of fluorescence over the equatorial region were classified as acrosome damaged.

Plasma Membrane Integrity Assessment: The protocol is adapted from the procedure described on stallion sperm by Vidament *et al.* (1998). 0.5 ml of sperm suspension diluted in HHBSA was centrifuged for 5 minutes at 590 g. Then, the final pellet was resuspended into a 50 mOsm HHBSA medium (without NaCl), and incubated for 15 minutes at 37°C. After the incubation period, one drop of semen suspension was placed over a pre-cleaned slide, and a cover-slip placed on top of the droplet.

A total of 100 spermatozoa were examined with a phase-contrast microscope for each sample. Interpretation was done according to Jeyendran *et al.* (1992). Straight or slightly curved tails were considered as not swollen. Swollen tails showed different degrees of curling or coiling.

Oocytes and Preparation of Hemizonae: Equine ovaries were collected from a slaughterhouse during the breeding season and transported to the laboratory in Phosphate-Buffered Saline solution (PBS) at room temperature within 4 hours after slaughter. Individual follicles were recovered by dissection of ovaries with the aid of surgical instruments and placed in plastic Petri dishes. Follicular walls were then cut, and cumulus-oocyte complexes were located under a stereomicroscope. Cumulus-oocyte complexes were vortexed to remove cumulus cells. The denuded oocytes were washed with PBS, then stored at 4°C in a 35 mm plastic Petri dish containing 0.5 ml of saline solution (1.5M $MgCl_2$, 0.1% (w/v) Polyvinylpyrrolidone

and 40mM Hepes buffer) (Coddington *et al.*, 1992), for at least 1 week until use for the hemizona assay.
On the day of the experiments, salt-stored oocytes were washed 4 times in Hepes-buffered Tissue Culture Medium TCM 199. Each oocyte was transferred to a new well containing 0.25 ml of culture medium, then immediately bisected manually under a stereomicroscope using hypodermic needles (Jean *et al.*, 1995). The ooplasm inside each hemizona was removed by scraping the inner side of hemizona once or twice with the needle. Each matched hemizona was placed in a separate droplet (50 μl) of culture medium in a 35 mm plastic Petri dish covered with mineral oil and kept at room temperature for 35 minutes until used for the sperm hemizona binding assay.

Sperm Preparation and Hemizona Binding Assay: For each ejaculate, 3 straws of each freezing extender were thawed in a 37°C water bath for 30 seconds. The contents of the 3 straws were pooled. Spermatozoa were washed 3 times with PBS and centrifuged each time for 10 min at 600g. After the last centrifugation, sperm pellets were re-suspended in a Tyrode-Lactate Medium modified for sperm treatment (SP-TALP, Parrish *et al.*, 1988) to a final concentration of 2.10^6 spermatozoa/ml. A 50 μl droplet of semen suspension was added to each hemizona and incubated for 4 hours at 39°C under 5% CO_2 in humidified air. Seventy oocytes were used. For each oocyte, one hemizona was treated with a Control sperm sample and the other one with a Glutamine sperm sample from the same ejaculate. Most of the ejaculates (12/15) were tested with 5 matching pairs of hemizonae, 3 ejaculates were tested with respectively 4, 3 and 3 matching pairs of hemizonae. After incubation, each hemizona was washed in culture medium by vigorous aspiration and expulsion four times through a narrow glass pipette, to dislodge loosely adherent spermatozoa. Washed hemizonae were mounted individually on a glass slide under a cover slip to flatten the hemizonae.
The total number of bound spermatozoa was counted under an inverted microscope fitted with a TV monitor. Because the two matching hemizona could not be exactly of equal size, a binding index (BI) was defined for each matched hemizona, to increase the reliability of the assay (Jean *et al.*, 1995). The surface of each matched hemizona was determined on a TV monitor covered with a transparent grid divided into $1cm^2$ squares (one square = one surface unit). The BI was defined as the total number of spermatozoa strongly bound to the hemizona x 100 / Total number of surface units of the hemizona (Fig. 1).
All chemicals were purchased from Sigma-Aldrich Chimie (St Quentin Fallavier, France).

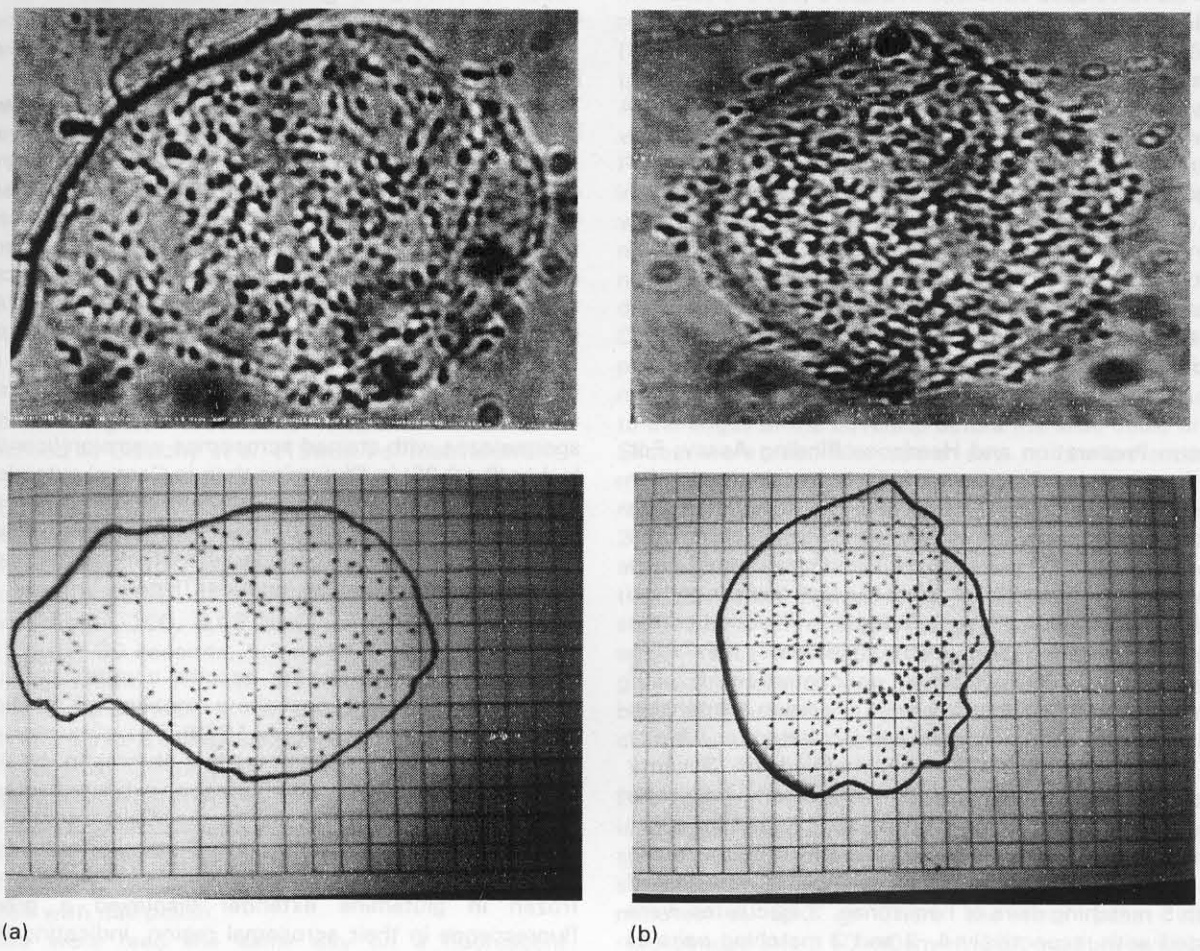
Statistical Analysis: Significant differences in values of post-thaw motility, nuclear and plasma membrane integrity, and acrosomal status, were estimated by the Wilcoxon test. Data from hemi zona assay were analysed by using Student paired t-test. A value of $P < 0.05$ was taken as statistically significant.

Results

The results are expressed as mean ± SEM. As shown in Table 1, motility after freezing and thawing was significantly higher ($P<0.001$) in the Glutamine extender than in the Control extender. For nucleus integrity, no significant difference between semen frozen in Control or Glutamine extender was observed, all the spermatozoa exhibited green fluorescence, showing more than 99% spermatozoa with intact DNA. In contrast, the percentage of swollen spermatozoa was significantly higher ($P<0.05$) in Glutamine than in Control extender. After exposure of permeabilized spermatozoa to FITC labeled PSA, the percentage of spermatozoa with stained acrosomes was significantly higher ($P<0.05$) in Glutamine than in Control extender. The results of the hemizona assay showed that BI was significantly higher ($P<0.05$)) for spermatozoa frozen-thawed in Glutamine extender than for spermatozoa frozen-thawed in Control extender (Fig. 1).

Discussion

In the present study, our results confirm that L-glutamine enhanced post-thaw motility of stallion spermatozoa (Trimeche *et al.*, 1999). The evaluation of structural stability of sperm nuclear chromatin, by AO staining, showed all spermatozoa exhibited green fluorescence in both freezing media. DNA integrity is thus maintained after freezing in Glutamine extender. We also observed that the majority of spermatozoa frozen in glutamine extender displayed a green fluorescence in their acrosomal region, indicating the presence of an intact acrosome. Moreover, we showed that the addition of 50mM glutamine in control extender preserved the plasma membrane integrity of frozen stallion spermatozoa more than using only glycerol as a conventional cryoprotectant. Similar results were obtained by Trimeche *et al.* (1998) for Poitou jackass frozen thawed spermatozoa in 3 freezing media: basal medium, basal medium + 80mM glutamine, basal medium + 10% quail egg yolk. In addition, stallion spermatozoa cryopreserved in glutamine extender were more able to bind to homologous zona pellucida than those frozen in control extender. This agrees with data from Trimeche *et al.* (1998) concerning the Poitou jackass. They also observed that 80 mM of glutamine in the freezing extender improved fertility, after insemination of Poitou jennies. Renard *et al.* (1996), reported that the addition of glutamine at 80mM in a basal medium further boosted the fertilizing capacity of frozen-thawed human spermatozoa, as estimated by use of the hamster egg penetration test.
We suggest that 50mM glutamine afforded more protection to functional characteristics of frozen-thawed stallion spermatozoa than the use of only glycerol in freezing extender.



(a) (b)

Fig. 1: Expression of strongly sperm-zona pellucida binding for spermatozoa frozen-thawed in (a) control and (b) glutamine extenders, in hemizona binding assay. For each hemizona, B.I. was defined as the total number of spermatozoa strongly bound to the hemizona x 100 / Total number of surface units of the hemizona

The evaluation of sperm motility using computer-assisted analysis systems (CASA) is not correlated with stallion fertility (Dowestt *et al.*, 1982; Voss *et al.*, 1982 and Jasko *et al.*, 1992). Moreover, motility after freezing and thawing is not strongly correlated with fertility when selected ejaculates are used (Palmer and Magistrini, 1992). Accordingly, sperm motility is not a reliable criterion to predict fertilizing capacity of frozen thawed semen. Therefore, combining the results of several sperm function tests improve the reliability of fertility estimation (Colenbrander, 2003).

The structure of sperm chromatin, as reflected by its sensitivity to thermal stress, may be an additional deterimant of fertility (Evason, 1980). Indeed, the freeze-thawed process could destabilize the relation between DNA structure and nuclear proteins, resulting in spermatozoa DNA that is surcondensated (Hammamah, 1991) and more susceptible to acid-induced denaturation. Consequently, this affects the rate of nuclear decondensation during fertilisation, and delays the segmentation cellular step, thereby disrupting embryo development (Perreault *et al.*, 1987). To date, DNA stability assessment using metachromatic proprieties of AO was used only to distinguish between fertile and subfertile stallions (Kenney, 1995; Love and Kenney, 1998). However, it is necessary to assess DNA stability of frozen-thawed spermatozoa because its fertilizing capacity does not end when spermatozoa are able to fertilizing an oocyte (to penetrate an oocyte), but also includes embryo development after fertilisation.

Mammalian spermatozoa undergo a delicate modification of the sperm membranes, before fertilisation. This event, termed capacitation, is required for sperm binding zona pellucida, then to undergo acrosome reaction for sperm-egg fusion (Yanagimamachi, 1994). Evaluation of acrosomal integrity using different techniques: (chlortetracycline (CTC) staining, Capacitation and Acrosomal Reaction (AR) assessing) were often used to assess the

Table 1: Effect of glutamine on functional characteristics of frozen-thawed stallion spermatozoa

	Control extender	Glutamine extender
Motility (%)[a]	35.2 ± 2,5	44.4 ± 3.5**
Nucleus integrity [a]	99.73 ± 0.2	99.93 ± 0.0*
Acrosomal status [a]	72.46 ± 1.7	74.86 ± 1.7*
Plasma membrane integrity [a]	66.40 ± 2.3	68.66 ± 2.7*
Binding Index [b]	143.86 ± 6.9	168.39 ± 8.3*

[a] N = 15 split ejaculates (3 stallions x 5 ejaculates)
[b] N = 70 matching hemizona
* significant difference (P < 0,05) between Glutamine and Control extender
** significant difference (P < 0,001) between Glutamine and Control extender

fertilizing capacity of fresh semen from fertile or subfertile stallions (Meyers *et al.*, 1995). However, the freeze-thaw process is an aggressive phenomenon, in which the sperm membranes undergo physical and biochemical damage (Amann and Pickett, 1987; Parks and Graham, 1992). If the acrosome is damaged during cryopreservation, it cannot bind to the zona pellucida of the oocyte. So, the evaluation of acrosome damage after the freeze- thaw process is an important parameter to consider. Colenbrander *et al.* (2003), reports that CTC staining, capacitation and Acrosomal Reaction assessing in unstimulated sperm are likely to be the most appropriate means of estimating the number of damaged sperm in a frozen-thawed sample, and there appears to be little difference in the rate of spontaneously acrosome reacted sperm in fresh semen from fertile or infertile stallions.

Plasma membrane integrity is essential for maintenance of cell viability and assessment of membrane function may be a useful indicator of the fertilizing ability of spermatozoa (Jeyendran *et al.*, 1992). However, cryopreservation of mammilan spermatozoa induced direct injuries on cellular membranes (Amann and Pickett, 1987 and Parks and Graham, 1992) which affected the fluidity of membranes and membrane ionic pumps such as Na^+/K^+ and Na^+/H^+, consequently causing great changes in osmotic pressure (Amman and Pickett, 1987 and Klug *et al.*, 1992). It is known that Na^+/K^+ ATP-ase is very important to regulating cell volume , both in increasing and decreasing pressure in ionic media (Darnell *et al.*, 1986). Additionally, Na^+/K^+ ATP-ase could be related to the response against hypo-osmotic shock of horse spermatozoa (Caiza de la Cueva, 1996). Therefore, the HOS test could be an indicator of the functioning of membrane ionic pumps and thier role in regulating osmotic pressure. So, the HOS test indicated not only whether the membrane is intact but whether it is osmotically active (Colenbrander, 2003). They also report that the HOS test may in fact be more appropriate for predicting the fertilizing capacity of frozen-thawed semen than fresh semen, given that membrane damage is a more important limiting factor in the former.

Sperm binding to the zona pellucida is an important preliminary step in the fertilization process, and reflects the multiple functions of spermatozoa such as viability, motility, acrosomal status (Franken *et al.*, 1989). The feasibility of HZA to provide 2 matching hemizona from the same oocyte, allowing a comparison of sperm binding, has been reported to distinguish between fertile and infertile stallions (Faseli *et al.*, 1995 and Pantke *et al.*, 1995). However, the HZA could be used not only as a clinical test but also as a functional test to assess the fertilizing ability of equine semen frozen in glutamine extender, as in our study.

To understand how glutamine may enhance the fertilizing capacity of frozen-thawed spermatozoa, a small number of studies have been published regarding the protective effects of amino acids and their mechanistic action at the cell level. Protection by amino acids, especially proline against freeze-thaw denaturation of the enzyme phosphofructokinase was shown by Carpenter *et al.* (1986). It was postulated that proline may intercalate between the phospholipid head groups of membrane lipids. Kruuve *et al.* (1988) and Kruuve and Glofcheski (1990) hypothesized that there is a glutamine-binding site on a plasmalemme-bound protein. Carpenter (1988) suggested that solutes and amino acids provide cryoprotection to a given protein. On the other hand, several studies have emphasized that prevention of lipid peroxidation is a result of the protective action of amino acids (Griveau *et al.*, 1995). They explained that this results in the protection of sperm enzymatic defence systems. In equine species, Trimeche *et al.* (1996) reported that glutamine acted at the extracellular level. Further experiments have to be conducted to confirm this mechanism.

Conclusion

In conclusion, our results demonstrate that the addition of glutamine at 50 mM in INRA freezing extender not only improved post-thaw motility, but also, preserved sperm chromatine, acrosome, and plasma membrane integrity, during the freeze-thaw process, and enhanced the ability of frozen-thawed stallion spermatozoa to bind to zona pellucida in vitro. All the functional tests described in the present study are commonly used to predict stallion fertility, nevertheless in vivo fertility remains to be tested. Further investigations are needed to understand the mechanism of action for glutamine at cellular and molecular levels.

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