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EFFECT OF SOME ANTIOXIDANTS ON ACROSIN AMIDASE ACTIVITY OF CHILLED STALLION SPERMATOZOA

Waheed M.M.* and El-Bahr S. M. **

Dept. of Clinical Studies* and Physiology, Biochemistry and Pharmacology** , College of Veterinary Medicine and Animal Resources, King Faisal University, Kingdom of Saudi Arabia.

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SUMMARY

The effect of some antioxidants on acrosin amidase activity of chilled stallion spermatozoa was investigated in this study. Nine ejaculates were collected from six Arabian horses. A comparison between four antioxidants namely, sodium pyruvate (0.5 mg/ml), sodium thiosulfate (STS, 1.0 mg/ml), bovine serum albumin (BSA, 5.0 mg/ ml), zinc chloride (0.15 mg/ml) and a mixture of them was studied in a chemically-defined stallion semen extender (Tris-egg yolk) at 5°C. The comparison was based on sperm viability, acrosin amidase activity and changes in the levels of extracellular alanine aminotransferase (ALT). Results of the present experiments revealed that sodium pyruvate and the mixture of antioxidants were most effective for improving viability and acrosin amidase activity of stallion spermatozoa. Low values of ALT in the extracellular medium were coincided with high values of acrosin amidase activity of equine stallion spermatozoa during storage at 5°C.

Keyword: Stallion, Semen, Acrosine amidase, Antioxidants

INTRODUCTION

Preservation of liquid semen at 5°C is an important technique in the breeding management of horses (Ball et al., 2001). Stallion spermatozoa have a dearth of endogenous antioxidants (by removing most seminal plasma) to protect them against reactive oxygen species (ROS) that may be present during hypothermic storage of semen (Ball et al., 2000). There is an evidence that oxidative damage of spermatozoa by ROS impairs stallion sperm function in terms of declined mo-

tility and fertility (Ball et al., 2001). Sodium pyruvate is well known as an antioxidant and has protective effects against hydrogen peroxide (Giandomenico et al., 1997). Furthermore, STS has been shown to be an antioxidant and calcium solubilizer, it produces its effect via increasing the production of hydrogen sulfide (Sen et al., 2008). Concerning BSA, it acts as a multifaceted antioxidant that binds copper and other metals and keeps them from participating in oxidation reactions (Gum et al., 2004; Bonoli-Carbognin et al., 2008). Also, BSA binds fatty acids and protects them from oxidation (Gum et al., 2004). The ability of zinc to retard oxidative processes has been recognized for many years (Powell, 2000). Zinc acts as antioxidant through protection of protein sulfhydryls or reduction of OH formation from H_2O_2 through the antagonism of redox-active transition metals, such as iron and copper (Powell, 2000). The supplement of extenders with antioxidants could induce beneficial effect on viability of chilled stallion spermatozoa (Waheed and Khalifa, 2004). Acrocin, a sperm-specific acrosomal proteinase, has an essential role in the fertilization process (Polankoski and Zaneveld, 1977; Kennedy et al., 1989). Low levels of acrosin in human semen appear to be associated with subfertility and infertility (Kennedy et al., 1989). The enzyme is localized in the rostral portion of the acrosome, but is absent from the equatorial segment (Garner et al., 1977). The total acrosin activity of ejaculates can not be predicted from standard semen parameters such as sperm concentration, motility and morphology (Goodpasture et al., 1987).

The main objective of the present study was to evaluate the effect of some antioxidants on acrosin amidase activity in stallion spermatozoa during storage at 5°C.

MATERIALS AND METHODS

Reagents

Benzamidine hydrochloride, N- a - benzoyl - DL-arginine p-nitroanilide hydrochloride (BAPNA) , dimethylsulfoxide (DMSO), HEPES (N-2-hydroxyethylpiperazin e- N' -2- ethanesulfonic acid), and Triton X-100 were obtained from Sigma Chemical Company (St. Louis, MO).

Solutions

Solution A. Detergent Buffer: 0.01% Triton X-IOO in 0.055 M HEPES, 0.055 M NaCl at 8.0. The solution is prepared by dissolving 1.31 g of HEPES, 0.32 g of NaCl and 1 ml of a 1% Triton X-IOO stock solution (1 ml Triton in 99 ml water) in 95 ml of distilled, deionized water. The solution is adjusted to pH 8.0 (with 1 N NaOH as required) and then adjusted to a final volume of 100 ml with distilled, deionized water. The detergent buffer is stable for three days but can be stored for extended periods in the refrigerator after addition of sodium azide (0.1%, or 100 mg added to 100 ml) as preservative.

Solution B. Benzamidine: 500 mM in water. The Solution is prepared by dissolving 87.3 g benzamidine-HCI in 1 liter distilled, deionized water. The solution can be stored in the refrigerator without any additives for at least two weeks.

Solution C. Substrate: 23 mM BAPNA in DMSO. The solution is prepared by dissolving 25 mg N-a-benzoyl -DL-arginine para-nitroanilide-HCI (BAPNA) in 2.5 ml dimethylsulfoxide (DMSO). The solution should be prepared fresh on the day of the assay. Complete dissolution of the BAPNA in DMSO requires about 5 to 10 minutes.

Solution D. Substrate-detergent mixture: 22.5 ml of the detergent buffer (solution A) is mixed thoroughly with 2.5 ml of the BAPNA/DMSO substrate solution (Solution C) in a 50-ml Erlenmeyer flask. This solution should be prepared while the Ficoll centrifugation step is taking place (see step 4 of the Assay Procedure).

Animals and ejaculates:

In this study, six healthy Arabian stallions (9 - 22 years old) belonging to three Arabian horse farms (Al-Bushaier Mydod, Haleim Shah and Al-Hashem farms), Al-Ahsa, Kingdom of Saudi Arabia, were used. These stallions have been used as sires in the regular breeding program of their farms.

Nine ejaculates were collected from the stallions using the CSU model artificial vagina. Immediately after collection, semen samples were transferred to the laboratory and evaluated by the conventional method.

Experimental design

In this investigation, comparison between four antioxidants namely, sodium pyruvate (0.5 mg/ml), STS (1.0 mg/ml), BSA (5.0 mg/ml), zinc chloride (0.15 mg/ml) and their respective mixture were done . The doses were used according to Waheed and Khalifa (2004). The comparison was based on evaluation of sperm viability, acrosin amidase activity in spermatozoa. In addition, the study was extended to determine the changes in the extracellular levels of ALT.

Experimental procedure

Following initial evaluation, each semen sample was prepared for preservation in Tris-egg yolk extender (Samper et al., 1988) at 5°C for 72 hours after being supplemented with the above mentioned antioxidants (Waheed and Khalifa, 2004). Briefly, 12 ml of each semen sample were divided into six aliquots (each one 2 ml). These aliquots were diluted with Tris-antioxidants free extender (Samper et al., 1988) 1:1 (v/v) and left for 5 minutes at 30°C. Samples were centrifuged at 1000 Xg for 5 minutes. Most of the supernatant was decanted and sperm pellets were resuspended in 2 ml of Tris-egg yolk extender supplemented with the antioxidants and reconstituted gently. The diluted samples were cooled slowly to 5°C (0.05-0.1°C/min) in the refrigerator and stored

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for 72 hours. Sperm progressive motility was assessed after dilution (0 hour) as well as after 6 and 72 hours of incubation period and viability indices were computed according to Milovanov et al. (1964).

Aliquot of one ml semen was removed after dilution and after 72 hours of incubation at 5°C. These aliquots were centrifuged (1000 Xg) and the supernatants were stored at - 20°C pending analysis. ALT (IU/L) was assayed spectrophotometrically under the conditions specified by the commercial kit systems (Reitman and Frankle, 1957).

Sperm pellets, following the removal of the supernatants, were stored at - 20°C until assay to evaluate the acrosin amidase activity (Kennedy et al., 1989). The assay procedure described in steps as follow:

1. The ejaculate (of known sperm concentration) is allowed to liquefy completely.
2. For each ejaculate, a volume is calculated that it will contain between 2 to 10 10^6 spermatozoa.
3. One control and 5 tests (contain each antioxidant) are preferably run simultaneously for each ejaculate. The control and testes are incubated in duplicates (0 and 72 hours).
4. 100 µl of the benzamidine solution (solution B) is immediately added to the control tube and the contents of the tube are mixed thoroughly by vortexing.
5. To each tube, including the control, 1 ml of the substrate-detergent mixture (solution D) is added and the contents of the tube are mixed thoroughly. Save at least 1 ml of solution E for step 9.
6. The tubes are incubated at 22 to 24°C for exactly three hours after addition of the assay solution. It is optimal to mix the contents of the tubes once every hour during the incubation period by vortexing.
7. After three hours of incubation, 100 µl of the benzamidine solution (solution B) is added to all tubes except the control.
8. All tubes are centrifuged at 1000 Xg for 30 minutes and the supernatant solutions are collected separately.
9. A spectrophotometer capable of holding 1-ml cuvettes is adjusted so that the substrate-detergent mixture (solution D) has an absorbance reading of 0.0 at 410 nm. Subsequently, the absorbance of each supernatant solution is recorded at 410 nm. The control solution should show no or almost no absorption.

Calculations

One IU of acrosin activity is defined as the amount of enzyme that hydrolyzes 1 µmol BAPNA/min at 23°C. To obtain whole numbers, the acrosin activity is expressed in µIU/10^6 spermatozoa. The activity is calculated simply by the following formula according to Kennedy et al. (1989).

As shown in Table 2, the rate of increase of ALT in the extra-cellular medium was significantly higher for controls (88.63%) in comparison to the use of antioxidants during storage of stallion semen at 5°C. Also, the lesser values of the rate of ALT increase in the extra-celluar medium were coincided by higher values of acrosin amidase activity in the stallion spermatozoa after 72 hours at 5°C (Table 2).

Table (2): The rate of increase of ALT (IU/L) in the extracellular medium and acrosin amidase activity in spermatozoa (uIU/10^6 sperm) during storage of stallion semen at 5°C (mean ± SEM).

Treatments	ALT after dilution	ALT after 72 hours at 5°C	Rate of ALT increase (%)	Acrosin activity
Control	12.23^{a} ± 0.93	23.07^{a} ± 0.40	88.63**	1.43^{ac} ± 0.39
Sodium pyruvate	20.25^{b} ± 1.65	29.51^{b} ± 1.13	45.73**	2.42^{a} ± 0.54
Sodium thiosulfate	18.08^{ab} ± 2.08	27.87^{ab} ± 2.52	54.14***	1.35^{ab} ± 0.29
BSA	15.18^{bc} ± 0.38	25.50^{ab} ± 0.93	67.98***	1.18^{bc} ± 0.32
Zinc chloride	14.31^{ac} ± 0.76	22.67^{a} ± 0.74	58.42***	1.35^{bc} ± 0.43
Respective mixture	18.66^{bc} ± 1.49	27.71^{ab} ± 1.98	48.50**	1.74^{b} ± 0.28

Means with dissimilar superscripts in the same column are significantly different at P<0.05

** p<0.01 *** p<0.001

Although, the correlation coefficient between the rate of ALT increase in the extra-cellular medium and acrosin amidase activity in spermatozoa was non-significant, there was a tendency of inverse correlation (r = - 0.55) .

DISCUSSION

Mammalian sperm contain particularly high concentrations of polyunsaturated fatty acids in their plasma membranes (Jones et al, 1979), making them susceptible to damage caused by lipid peroxidation (Jones and Mann, 1977). Lipid peroxidation of spermatozoal membrane lipids has been reported to be the major cause for the loss of sperm motility and fertilizing ability in human spermatozoa incubated for long periods of time during in vitro fertilization procedures (Aitken et

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al., 1989; Gomez and Aitken, 1996), by altering membrane fluidity and functional integrity (Aitken, 1995) as a result of ROS accumulation. Although cooling spermatozoa to 5°C reduces the rate of lipid peroxidation, it is likely that lipid peroxidation plays a significant role in the reduced fertility seen for stallion spermatozoa stored for long periods of time (Pickett et al., 1989). Hypotaurine, taurine, and glutathione are antioxidants found in stallion semen (Li, 1975; Bavister and Yanagimachi, 1977). Antioxidants are added to reduce ROS formation and lipid peroxidation, thereby increasing spermatozoal longevity (Aitken, 1995). Interestingly, till the time of the present study, there were no publications concerning the effect of antioxidants on acrosin amidase activity in stallion spermatozoa and for this reason the present study was performed.

Concerning the effect of antioxidants on sperm motility and viability, the present findings indicated that, antioxidants improved stallion sperm motility and viability during incubation at 5°C (Table 1). Similar results have been previously reported in human (De Lamirande and Gagnon, 1992; Alvarez and Storey, 1995; Gavella and Lipovac, 1998; Kouchi et al., 1999), rams (Olero et al., 1998; Upreti et al., 1998), bulls (Bilodeau et al., 2002) and stallions (Bruemmer et al., 2002; Waheed and Khalifa, 2004). Pyruvate is a potent scavenger of H_2O_2 (Upreti et al., 1998) and its supplementation at a concentration of 5 mM (~0.55 mg ml^{-1}) to chilled-stored stallion semen resulted in a significant augmentation of sperm motility and ATP levels (Bruemmer et al., 2002).

Oxidation of thiols (SH) in sperm proteins by O_2^- and H_2O_2 was found to be associated with inhibition of sperm motility and fertilizing ability (Mammoto et al., 1996). Supplementation of egg yolk extender with sodium thiosulfate (1 mg ml^{-1}), as a source of SH to sperm cells, led to a remarkable improvement of their motility and plasma membrane stability in buffalo semen (Khalifa, 2001). Luxuriant entry of Ca^{2+} into sperm cells was found to coincide with activation of membrane-bound phospholipases (Roldan, 1998) and peroxidative damage of their membranes (Megshed and Kumar, 1997). The stabilizing influence of zinc on sperm membranes was attributed to its ability to suppress lipid peroxidation via inhibition of phospholipases and to protect SH and poly unsaturated fatty acids in biomembranes from Fe^{2+}- mediated oxidation (Andrews et al., 1994). Albumin is a crucial extracellular antioxidant (Alvarez and Storey, 1983) through its propensity to bind transition metal ions (Fe^{2+} and Cu^{1+}) in egg yolk extender and, thereby, to minimize formation of OH, the powerful initiator of lipid peroxidation cascade in sperm (Halliwell, 1988).

Regarding the effect of antioxidants on acrosine amidase activity, the current study revealed that, sodium pyruvate and the antioxidants mixture preserved the acrosin amidase activity in stallion

spermatozoa during liquid storage at 5°C in Tris-egg yolk extender (Table 1). On the same direction, Ball et al. (1997) found that addition of egg yolk to equine spermatozoa significantly increased acrosin amidase activity in the cryopreservation medium. Furthermore, if the spermatozoa are damaged by cold shock, the proacrosine undergoes activation within the inner acrosomal membrane and acrosin appears in the extract instead of proacrosin (Mann and Lutwak-Mann, 1981).

The superior effect of Pyruvate than the other antioxidants on acrosine amidase activity preservation (Table 1) perhaps attributed to that pyruvate is an effective energy substrate as it can freely move into the cytoplasm and the mitochondria of cells reducing the ROS at a rate higher than the other used antioxidants. However, with the exception of pyruvate, the antioxidants mixture performed better than the individual use of antioxidants at the level of acrosine amidase activity. Interestingly, pyruvate alone preserved the acrosine amidase activity better than that when it was mixed with the other antioxidants. This perhaps indicated some sort of antagonism between pyruvate and the rest of antioxidants used.

In view of our findings, antioxidants offered a better protection for stallion sperm during storage at 5°C than the antioxidant free extender (Table 2). This is confirmed by the increased rate of extra-cellular ALT in the antioxidant free extender (Table 2). Pursel et al. (1968) reported that one of the consequences of acrosomal damage is the leakage of enzymes from the sperm. The leakage of ALT revealed a positive correlation between enzyme release and sperm cell integrity and acrosomal damage (Chauhan et al., 1993). Enzyme release has generally been recognized as an indicator of cellular injury whereby membranes become inactivated or destroyed resulting in the loss of cellular material (De Reuck and Knight, 1964). Positive correlation between enzyme release and acrosomal damage was reported with goat's sperm (Chauhan et al., 1993). Yousef and Zeitoun (1998) found that there were negative correlation coefficients between sperm motility on one side and ALT release on the other side. They reported that the activities of these enzymes could be used as an indicator of sperm integrity. Concerning the effect of antioxidants as decreasing the activity of ALT, similar results were reported previously (Waheed and Khalifa, 2004). Further, the results of this study provide clear evidence that antioxidants protect the stallion spermatozoa against damage during cold storage and subsequently keep the acrosin amidase activity with spermatozoa. This is elucidated by the significant ($p<0.01$) decrease in the rate of extracellular ALT that coincided with high values of acrosin amidase activity in stallion spermatozoa after cold storage.

Accordingly, acrosin is an indicator of sperm activity (Zaneveld et al., 1996). The present study

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concluded that acrosin activity of spermatozoa may be potentially used as a useful marker of semen quality .

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