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INFLUENCE OF PENTOXIFYLLINE ON VIABILITY AND FERTILITY OF PRESERVED RAM SPERMATOZOA

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ABSTRACT

The effect of pentoxifylline on sperm viability and fertility was investigated after hypothermic preservation of ram semen in a liquid or frozen form. Prestorage fortification of Tris-based extenders with 0.25, 0.50, 1.00 and 3.00 mM pentoxifylline significantly ($P < 0.05$) augmented the survival of spermatozoa at 5°C compared with control extenders or extenders fortified with 5.00, 10.00, 15.00 and 20.00 mM pentoxifylline. Whereas, post thaw supplementation of cryopreserved (in the form of pellets) ram semen with 15.00 mM pentoxifylline did minimize the rapid deterioration (within 3 hours of post thaw incubation at 30°C) in sperm motility without any valuable improvement in pregnancy rates of cervically inseminated ewes.

Keywords: pentoxifylline, spermatozoa, preservation, ram.

INTRODUCTION

Artificial insemination (AI) and accurate progeny testing schemes can substantially increase the rate of genetic progress of economically important traits in animal breeding technology (Evans and Maxwell, 1987). However, AI in sheep has not been widely adopted due to the relatively poor fertility obtained when frozen-thawed semen is used for intracervical insemination (Maxwell *et al.*, 1995a and Söderquist *et al.*, 1997). Consequently, various compounds have been used with the objective of improveing in viability and fertility of ram spermatozoa (Salamon and Maxwell 1995 a, b and Maxwell and Watson, 1996 and Upreti *et al*., 1998). Pentoxifylline (a methylxanthine phosphodiesterase inhibitor) could stimulate sperm motility (by increasing the intracellular levels of cAMP) and might have a potential inhibitory effect (as an antioxidant) on production of reactive oxygen species - induced lipid peroxidation that involved in the mechanisms by which spermatozoa were damaged in many cases of male infertility (Gavella and Lipovac, 1992 ; Maxwell *et al.*, 1995 b; Mckinney *et al.*, 1996 and Okada *et al.*, 1997). Since the low fertility obtained after cervical insemination with preserved semen is primarily due to failure of establishment of an adequate cervical population of spermatozoa, which is associated with impaired sperm transport in the ewe's genital tract (Salamon and Maxwell, 1995 b), we hypothesized in this study that pentoxifylline might able to provide a sufficient duration of motility stimulation for spermatozoa to increase the number of sperm migrating through cervical mucus as well as the number of sperm reaching the site of fertilization in the upper reproductive tract of female.

MATERIALS AND METHODS

Animals and Semen Collection

Semen was collected with an artificial vagina from five crossbred rams (3/8 Finnish Landrace × 5/8 Rahmani) aged 3-3.50 years from September to October, 1999. All these animals were kept at Sakha Experimental Station, Animal Production Research Institute. Semen samples were assessed before beginning the study, and ejaculate volume, initial sperm concentration, motility, and morphology were all within the respective normal ranges for rams according to the standards at the semen laboratory of the Department of Theriogenology at Faculty of Veterinary Medicine, Cairo University (Abou-Ahmed, 1982).

Semen Extender

A Tris-based diluent was used to extend ram semen (Evans and Maxwell, 1987). It was composed of Tris (hydroxymethyl) amino methane (3.634 g), glucose (0.500 g), citric acid monohydrate (1.990 g), fresh chicken egg yolk (15 ml), glycerol (5 ml), penicillin (100,000 IU), streptomycin (100 mg), and glass-distilled water to 100 ml.

Semen Processing

Experiment 1:

This experiment was conducted to investigate the effect of pentoxifylline (Sigma - Aldrich Co., Deisenhofen, Germany) on sperm viability during short - term storage of ram semen at 5°C. Only ejaculates of good initial motility (80 - 90 %) and sperm concentration (at least 2.50×10^9 /ml) were used in this experiment. On each occasion, 2 to 3 consecutive ejaculates were collected from each ram during a period of approximately 5 to 10 minutes. The pooled ejaculates from each ram, with a total volume of 2.0 to 3.0 ml, were then split and diluted at 30°C (1 part semen + 19 part extender) with Tris-based extenders supplemented with 0 (control), 0.25, 0.50, 1.00, 3.00, 5.00, 10.00, 15.00 and 20.00 mM pentoxifylline. Immediately after dilution, the extended semen was placed in the refrigerator and incubated at 5°C for 168 hours. In the above-mentioned experiment, 20 trials were made to test the effect of pentoxifylline on post-treatment sperm survival. Sperm motility was assessed subjectively (at o {after dilution}, 6, 24, 48, 72, 96, 120, 144, and 168 hours) using a phase-contrast microscope (40 X) equipped with a thermal stage at 37°C. The viability index of incubated semen was calculated according to Milovanov *et al.* (1964).

Experiment 2:

This experiment was carried out to monitor the effect of pentoxifylline on sperm viability after freeze / thaw processing of ram semen by the pellet method. Accordingly, a total number of 25 ejaculates were collected from the five rams (5 ejaculates from each ram). Immediately after semen collection, the volume was measured, and aliquots of raw semen were taken for determination of sperm motility and sperm concentration in a

haemocytometer, according to the method described by Bane (1982). Only ejaculates of 70 to 80 % initial motility and at least 2.5×10^9 sperm cells / ml semen were diluted (1 part semen + 4 parts extender) at 30°C with a single addition of a Tris - based extender. The extended semen was cooled to 5°C in 1.5 - 2.0 hours (Maxwell *et al.*, 1995a). The cooled semen was frozen in pellet form (0.30 ml / pellet) on a special plastic plate with holes engraved in the surface and cooled to - 79°C to - 140°C by immersion in liquid nitrogen (Evans and Maxwell, 1987). After 2 to 3 minutes, the frozen pellets were transferred into liquid nitrogen and stored for 3-60 days before thawing. For each freezing trial, a thawing solution (supplemented with or without 15.00 mM pentoxifylline) was used for thawing of frozen pellets (Maxwell *et al.*, 1995 b). It was composed of Tris (hydroxymethyl) amino methane (3.634 g), glucose (0.500 g), citric acid monohydrate (1.990 g), and glass - distilled water to 100 ml. Thus, two to three pellets were dropped into a prewarmed (at 40°C for 15 minutes) glass 10-ml test tube containing 0.50 ml of thawing solution held in a water bath at 40°C. The tube was gently shaken until melting of pellets. Sperm motility was assessed (as previously mentioned in Exp.1) immediately after dilution, before freezing and after thawing. The post thaw recovery rate (%) was calculated according to the following formula:

$$\frac{\text{Sperm motility immediately after thawing}}{\text{Sperm motility immediately after dilution}} \times 100$$

In addition, aliquots of extended semen (immediately before freezing and after thawing) were transferred to clean, narrow, prewarmed glass vials, and incubated in a water bath at 30°C for 3 hours. Sperm motility was subjectively assessed at 0, 1, 2 and 3 h of incubation period and the rate (%) of reduction in sperm motility was calculated according to the following formula:

$$\frac{\text{\% motile sperm at 0 hour - \% motile sperm at 3 hours}}{\text{\% motile sperm at 0 hour}} \times 100$$

Also, the post thaw viability index was estimated according to Milovanov *et al.* (1964).

Fertility Test

Design:

To detect if the difference in post thaw sperm viability observed in Exp. 2 due to pentoxifylline treatment would be reflected in difference in fertility, individual ejaculates from 2 rams were frozen in the form of pellets. After thawing, each pellet (0.30 ml) contained 90 - 100 x 10^6 progressively motile spermatozoa.

Experimental animals and synchronization of estrus

A total number of 60 pluriparous crossbred ewes (3/8 Finnish Landrace × 5/8 Rahmani)with an average body weight of 51.4 kg were used in the fertility test which commenced in January, 2000 and lasted for one

month. Each ewe received two intramascular injections (with a 11 day injection interval) of 1ml (7.50 mg luprostiol) synthetic prostaglandin $F_{2\alpha}$ (Prosolvin®, INTERVET). After two days of the second injection, the ewes were placed with vasectomized rams (10: 1, ewes: rams) for drafting of estrous females.

Insemination and pregnancy diagnosis :

Two frozen semen pellets were thawed in a thawing solution (0.50 ml) supplemented with or without 15.00 mM pentoxifylline according to the procedures described in Exp. 2. Thawed semen was held in a water bath (30°C) and was used for insemination within 10 to 15 minutes. An inseminate volume of 1.10 ml (containing 180 - 200 x 10^6 progressively motile spermatozoa) was used for double cervical insemination of the ewes. For each semen treatment, the first insemination was performed immediately after estrous detection and the second insemination was performed 8 hours after estrous detection. For insemination, the hindquarters of females were raised over a rail, and the semen was deposited as deeply as possible into the cervical canal using a simple inseminating pipette with a bent tip and a duck - bill speculum. After 50 days of insemination, the ewes were screened for pregnancy by transabdominal ultrasonography.

Statistical Analyses:

All data from Experiment 1 and 2 were subjected to analysis of variance (ANOVA) according to the statistical procedures that were reported by Snedecor and Cochran (1980), to clarify the effect of:

* Semen treatments and incubation periods on sperm motility and viability index (Exp. 1).
* Semen treatments on post thaw sperm motility, recovery rate, viability index and rate of reduction in sperm motility (Exp. 2).

Treated means were compared by the least significant difference test at 5% level of probability. The difference in pregnancy rate among semen treatments were analyzed by the Chi-square (χ^2) test.

RESULTS

Experiment 1:

Table 1 depicts the effect of different concentrations of pentoxifylline on sperm motility and viability of chilled ram semen. It is evident that the motility and viability of spermatozoa exposed to 0.25, 0.50, 1.00 and 3.00 mM pentoxifylline were significantly higher ($P < 0.05$) than that exposed to 0 (control), 5.00, 10.00, 15.00 and 20.00 mM pentoxifylline. This effect became more pronounced after hypothermic storage of semen for 72 and 168 hours. Irrespective of semen treatments, there was a dramatic decrease ($P < 0.05$) in sperm motility after incubation of ram semen for 72 and 168 hours at 5°C.

Experiment 2:

Table 2 outlines the influence of pentoxifylline (15.00 mM) on post thaw survival of ram spermatozoa. There was no significant difference in post thaw motility and recovery rate of spermatozoa between semen treatments. However, the post thaw viability index was significantly higher ($P < 0.05$) for semen treated with pentoxifylline than that for untreated (control) semen.

Comparison was made between rates of reduction in sperm motility during in vitro incubation (at 30°C for 3 hours) of cooled semen (before freezing) and frozen / thawed semen. The mean rates of reduction in sperm motility for cooled semen, frozen / thawed semen and frozen/thawed semen fortified with 15.00 mM pentoxifylline were 27.64%, 36.45% and 26.95%, respectively ($P < 0.05$).

Table 1: Influence of pentoxifylline on motility (%) and viability of ram spermatozoa during incubation at 5°C (Mean ± SE).

Pentoxifylline Concentrations	Incubation periods (hours)				Overall means	Viability Index
	0	6	72	168		
Control	85.00 ^{a} ± 0.98	79.67 ^{a} ± 0.77	67.67 ^{a} ± 1.53	21.67 ^{a} ± 1.52	56.33 ^{ab} ± 3.84	99.06 ^{a} ± 2.11
0.25 mM	86.00 ^{a} ± 1.87	83.00 ^{a} ± 2.00	70.00 ^{ab} ± 3.54	28.00 ^{bcd} ± 2.00	66.75 ^{abcd} ± 5.43	112.68 ^{b} ± 4.24
0.50 mM	85.00 ^{a} ± 1.58	84.00 ^{a} ± 1.00	75.00 ^{ab} ± 1.58	30.00 ^{bcd} ± 1.58	68.50 ^{abcd} ± 5.22	116.97 ^{b} ± 1.03
1.00 mM	87.00 ^{a} ± 1.33	84.50 ^{a} ± 1.74	75.00 ^{ab} ± 1.83	33.50 ^{bcd} ± 1.83	70.00 ^{abcd} ± 3.50	118.98 ^{b} ± 3.39
3.00 mM	90.00 ^{a} ± 0.00	90.00 ^{b} ± 0.00	74.00 ^{ab} ± 1.00	28.00 ^{bcd} ± 2.00	70.50 ^{abcd} ± 5.85	111.12 ^{b} ± 1.89
5.00 mM	88.50 ^{a} ± 0.76	85.50 ^{a} ± 1.17	52.50 ^{b} ± 2.71	8.00 ^{bc} ± 0.82	58.63 ^{ab} ± 5.19	76.11 ^{bc} ± 2.36
10.00 mM	83.00 ^{a} ± 1.22	78.00 ^{a} ± 1.22	39.00 ^{bc} ± 3.32	5.00 ^{bc} ± 0.00	51.25 ^{abc} ± 7.32	57.54 ^{bcd} ± 3.58
15.00 mM	90.00 ^{a} ± 0.00	80.00 ^{a} ± 0.00	20.00 ^{bcd} ± 2.74	6.00 ^{bc} ± 1.00	49.00 ^{bc} ± 8.40	41.85 ^{bcde} ± 2.53
20.00 mM	90.00 ^{a} ± 0.00	77.00 ^{a} ± 1.30	20.00 ^{bcd} ± 1.21	5.00 ^{bc} ± 0.00	40.00 ^{bc} ± 6.41	39.81 ^{bcde} ± 2.98
Overall means	86.67 ^{A} ± 0.48	82.83 ^{A} ± 0.61	61.33^{B} ± 2.27	26.25^{BC} ± 1.3	62.81 ± 1.84	93.96 ± 3.30

Means with different alphabetical superscripts A, B and C for the last row or a, b, c, d, and e for columns are significantly different ($P < 0.05$).

Table 2: Influence of pentoxifylline on post thaw survival of ram spermatozoa (Mean ± SE).

Semen treatments	Sperm motility (%)			Recovery rate (%)	Post thaw viability index
	After dilution	Before freezing	After thawing		

Control	74.40 ± 0.78 (25)	70.40 ± 0.96 (25)	48.00[a] ± 1.53 (25)	64.59[a] ± 2.01 (25)	130.80[a] ± 6.02 (25)
15.00 mM Pentoxifyllin			50.60[a] ± 1.27 (25)	68.08[a] ± 1.70 (25)	149.30[b] ± 6.67 (25)

- **Means with different superscripts in the same column are significantly different (P < 0.05).**
- **Figures in parentheses denote number of ejaculates involved .**

The outcome for cervical insemination of estrous ewes with frozen/thawed semen is shown in Table 3. The pregnancy rates of untreated and pentoxifylline- treated semen were 36.67% and 43.33%, respectively. Chi-square analysis did not reveal any significant difference in pregnancy rate among post thaw semen treatments.

Table 3:Fertility of frozen / thawed ram spermatozoa after in vitro supplementation with pentoxifylline.

Semen treatments	Number of inseminated ewes	Number of pregnant ewes*	Pregnancy Rate (%)
Control	30	11	36.67
15.00 mM pentoxifylline	30	13	43.33

***Pregnancy diagnosis by ultrasound 50 days after insemination.**

DISCUSSION

Since the earliest observations on hypothermic preservation of ram semen, it has been realized that the reduction and increase in temperature inevitably causes ultrastructural, biochemical and functional damage to a significant proportion of spermatozoa (Salamon and Maxwell, 1995 b). These changes are accompanied by a decrease in viability and fertility (after cervical insemination) of ram semen that has been stored in a liquid (for more than 24 hours) or frozen state (Evans and Maxwell, 1987 and Maxwell and Watson, 1996). In this connection, comprehensive studies were conducted on methods and attempts to improve sperm viability (Fernandez *et al.*, 1985 and Robinson *et al*., 1992) and sperm transport (Campbell *et al.*, 1996 and Sayre and Lewis, 1997) in the ewe's genital tract. However, despite increases in the percentage of motile spermatozoa in the preserved ram semen have been reported after addition of the methylxanthines, these increases in motility have not yet been reflected in increased fertility (Salamon and Maxwell, 1995 b). Concomitantly, the current study indicated that prestorage incorporation of pentoxifylline (0.25, 0.50, 1.00 and 3.00 mM) in ram semen extenders was able to maintain a high percentage of viable spermatozoa during in vitro

incubation at 5°C for more than 72 hours. This enhancement in the quality of stored spermatozoa might be due to pentoxifylline at these concentrations could minimize (as an antioxidant) the potentially toxic and spermicidal effects of reactive oxygen species (McKinney *et al*., 1996 and Okada *et al*., 1997) that produced by dead spermatozoa during short - term storage of ram semen in egg yolk - based extenders (Upreti *et al.*, 1994 a, b).

On the contrary, it was observed form the present study that in vitro incubation (at 5°C) of ram semen with higher concentrations (5.00, 10.00, 15.00 and 20.00 mM) of pentoxifylline dramatically reduced the viability of spermatozoa compared with untreated semen. In this respect, a variety of postulates in the published literature pointed out the deleterious influence of pentoxifylline at a certain concentration on sperm viability. Acosta and Kruger (1996) attributed the rapid decline in motility of human sperm samples to a toxic effect of methylxanthines on the sperm ultrastructure, especially damage to the sperm plasma membrane, or to an accelerated rate of capacitation, since on completion of capacitation sperm underwent the acrosome reaction and had a shorter life span than non-reacted sperm. The latter assumption is ruled out and unlike the findings of our experiment owing to the presence of decapacitation factors (such as seminal plasma and egg yolk) in the incubation media (Cross, 1998 and Visconti and Kopf, 1998). In addition, the presence of glucose and other glycolyzable substrates in ram semen extenders might delay or inhibit capacitation of spermatozoa (Parrish *et al.*, 1989). On the other hand, Nivsarkar *et al.*, (1998) recorded a significant augmentation of lipid peroxidation with a concomitant reduction in sperm viability after in vitro treatment of human semen with pentoxifylline. However, it is not plausible to accept the claim of latter authors as a rational justification for results of our experiment because they conducted their experiments on oligozoospermic semen samples suffered from preaccumulation of lipid hydroperoxides in sperm plasma membrane. In the light of above-mentioned arguments, we can speculate that the decrease in viability of stored ram spermatozoa after their exposure to 5.00 - 20.00 mM pentoxifylline might be due either to disruption of sperm plasma membrane integrity or to the harmful effect of reduced extracellular and intracellular pH on sperm survival that induced by over accumulation of toxic waste products (mainly lactic acid) from the progressive increase in rate of sperm glycolysis at the expense of oxidation rate of lactate and pyruvate to CO_2 without any marked degree of perturbation in the intracellular concentrations of ATP and ADP (Rees *et al.*, 1990).

Previous disputations had shown that the antioxidant defense system in mammalian spermatozoa was comparatively weak (Vishwanath and Shannon, 1997) and these cells were very susceptible to oxidative stresses during the cycle of freeze/thaw processing of ram (Nauck, 1988 and Erochin and Derjazencev, 1991), bull (Bilodeau and Bras, 1999 and Bilodeau *et al.*, 1999), equine (Ball, 1999) and human (Bell et al., 1993) semen. The latter authors detected that the freeze / thaw process could reduce glutathione (GSH) level and superoxide dismutase (SOD) activity in spermatozoa with a subsequent elevation of reactive oxygen species (mainly superoxide anions, hydroxyl radicals and hydrogen peroxide) in thawed semen. Furthermore,

after thawing, the cryopreserved ram (Perez *et al.*, 1996) and bull (Collin and Bailey, 1999) spermatozoa take - up calcium ions faster than fresh spermatozoa and this intracellular calcium increases could stimulate a calcium-dependent burst of reactive oxygen species (Aitken and Clarkson, 1987). It had been proposed that reactive oxygen species (ROS) did initiate the sequence of sperm capacitation and the undesirable membrane destabilization associated with frozen storage of ram spermatozoa could be inhibited or reversed by using antioxidants (Maxwell and Watson, 1996). Supporting the above-stated observations and in contrast to the findings of Maxwell *et al.* (1995 b), the current investigation proved that post thaw supplementation of cryopreserved ram semen with 15.00 mM pentoxifylline significantly maintained sperm viability (Table 2) and minimized the rate of post thaw reduction in sperm motility to a level mimiced the rate of reduction in sperm motility of cooled unfrozen semen.

Because lipid peroxidation is a major factor limiting the longevity of mammalian sperm (Mann and Lutwak - Mann, 1981 and Windsor, 1997), it is possible to envisage that the tendency of pentoxifylline to scavenge ROS (McKinney *et al*., 1996 and Okada *et al*., 1997) might have extended the viability of frozen / thawed ram semen by inhibition of premature capacitation and death of spermatozoa (Thomas *et al.*, 1998).

An intriguing feature of the fertility experiment was the slight non-significant increase in the pregnancy rate of pentoxifylline - treated semen relative to control after cervical insemination of estrous ewes. It was suspected from our in vitro experiment that pentoxifylline was able to extend the survival of sperm in the female genital tract by improvement of the preferential binding of uncapacitated sperm to oviductal epithelial cells (Fazeli *et al.*, 1999). Since our study was carried out on a small population, future trials using a larger group are needed in order to definitely conclude that pentoxifylline can improve the fertility of frozen / thawed ram semen.

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تـأثير البنتوكسـى فللـين علـي حيويـة وخصـوبة الحيوانـات المنويـة المحفوظـة فـي الكباش

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استهدفت الدراسة قياس تأثير إضافة البنتوكسي فللين على حيوية وخصوبة الحيوانات المنوية المحفوظة في الكباش.

تم هذا البحث في تجربتين :-

التجربة الأولي :- كان الهدف منها دراسة تأثير إضافة البنتوكسي فللين بمستويات مختلفة علي حيوية الحيوانات المنوية خلال الحفظ علي درجة 5 م[5] لمدة 168 ساعة .

وكانت من نتائج هذه التجربة أن إضافة البنتوكسي فللين علي السائل المنوي المخفف بمخفف الترس بمستويات 0.25 ، 0.50، 1 ، 3 مل مول يزيد معنوياً من حيوية الحيوانات المنوية وهذا التأثير يكون أكثر تحققاً بعد 72 ، 168 ساعة من الحفظ على درجة 5م[5] مقارنةً بعينات السائل المنوي الغير معامل (كنترول) وكذلك عينات السائل المنوي المضاف إليه البنتوكسي فللين بمستويات 5 ، 10 ، 15 ، 20 مل مول.

التجربة الثانية :- كان الهدف منها دراسة تأثير إضافة البنتوكسي فللين علي حيوية وخصوبة الحيوانات المنوية بعد إسالة السائل المنوي المجمد علي شكل حبيبات .

وكانت من نتائج هذه التجربة أنه لا توجد اختلافات معنوية في حيوية الحيوانات المنوية بعد الإسالة مباشرةً للسائل المنوي المعامل (المضاف إليه 15 مل مول بنتوكسي فللين) وغير المعامل (كنترول) . إلا ان المعاملة قد خفضت من الانخفاض في حيوية الحيوانات المنوية خلال 3 ساعات من الإسالة والحفظ علي درجة 30م[5] مقارنةً بعينات السائل المنوي غير المعامل بدون أي تحسن معنوي في نسبة الخصوبة في النعاج الملقحة عن طريق عنق الرحم .