

The effect of oestradiol-17 β on the motility, viability and the acrosomal status of bull sperm

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Abstract

The aim of this study was to test the effect of oestradiol-17 β (E₂) on the motility, viability and the acrosomal status of bull sperm *in vitro*. Pooled semen from Holstein bulls were incubated in the presence of 2, 4, and 8 μ g E₂/mL for 24 h. Semen was also incubated in media without E₂. During the incubation, the number of motile, viable sperm and the number of sperm possessing lost/damaged acrosomes, cytoplasmic droplets and coiled tails were counted at 0, 4, 18 and 24 h of incubation. Addition of 2 μ g E₂/mL at 18 h of incubation increased the total motility over the control. The number of forward progressing sperm was increased by the supplementation of 2 and 8 μ g E₂/mL over the control group at the 4 h incubation. Lower doses of E₂ (2 and 4 μ g/mL) did not affect viability of sperm, but a high dose of E₂ (8 μ g/mL) caused reductions in viability at 4 and 24 h of incubation. The number of sperm cells with lost acrosomes was significantly high in control group at 24 h of incubation. The number of sperm cells possessing proximal and distal cytoplasmic droplets and the number of sperm cells bearing coiled tails were not altered by any of the treatments. A small dose of E₂ (2 μ g/mL) had a beneficial effect on the motility and acrosome integrity of bull sperm *in vitro*. Higher dose of E₂ (8 μ g/mL) had a detrimental effect on viability.

Keywords: Bovine males, estradiol, acrosome, motility, spermatozoa, *in vitro* incubation

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Introduction

Environmental oestrogens display oestrogen-like activity. Their effects on mammalian sperm function have already been shown (Adeoaya-Osiguwa *et al.*, 2003). Decrease in fertility in aromatase knock-out male mice was severe when the animals were maintained on a soya-free diet (O'Donnell *et al.*, 2001; Robertson *et al.*, 2002). The most potent oestradiol is oestradiol-17 β (E₂) which is synthesized through the aromatization of testosterone by the enzyme P₄₅₀ aromatase in the testis (Rommerts *et al.*, 1982; Tsai-Morria *et al.*, 1985). It plays an important role in reproduction by regulating the function of the efferent ductules and the epididymis where sperm mature and gain fertilizing ability (Hess *et al.*, 1997; Carreau *et al.*, 2003; Hess & Carnes, 2004; Lukoseviciute *et al.*, 2005).

The presence of oestrogen receptors (ER α and β) has been shown within the male testes of many species except bulls. Therefore, not enough information is available about the action of oestrogens on bull sperm. It has been reported that oestrogen regulates re-absorption of luminal fluid in the head of the epididymis. Disruption of this function causes sperm to enter the epididymis diluted (Hess *et al.*, 1997). This probably results in morphological abnormalities, leading to infertility. In female animals the concentration of E₂ increases during the oestrus and follicular rupture. After insemination, sperm would be exposed to E₂, particularly in tubal fluid. Presence of E₂ in the female reproductive tract during this time modulates the motility and extent of the viability of sperm (Hunter, 1988).

Motility of sperm is one of the primary mechanisms of sperm transport and traits closely correlated with fertility. Sperm cells can be transported efficiently only in females in oestrus or upon administering E₂ to ovariectomized females (Allison & Robinson, 1972; Bedford, 1972; Hunter, 1988; Boquest & Summers, 1999). The effect of E₂ on motility of sperm was studied extensively in humans (Cheng & Boettcher, 1981; Idaomar *et al.*, 1989; Allag & Rangari, 1997; Wang *et al.*, 2001; Lambard *et al.*, 2003) and rodents (Bandophyaha *et al.*, 1974; Hawk & Cooper, 1976; Das, 1977; Rao & Mathur, 1988; Jin *et al.*, 2005). There are a few reports on the use of E₂ in *in vitro* experiments with sheep. This is because the majority of studies concerning the effect of E₂ in sheep focused on the transfer of sperm in the female reproductive tract (Croker *et al.*, 1975; Hawk & Cooper, 1975; Hawk *et al.*, 1978). In bulls the majority of *in vitro* studies focused

principally on the interaction between E_2 and oviductal cells and the influence of E_2 on sperm motility, viability as well as on hormonal secretion (Boquest & Summers, 1999; Wijayagunawardane *et al.*, 1999; Martinez *et al.*, 2005).

Limited studies have been performed to monitor *in vitro* the effect of oestrogens on bull sperm. Therefore, the objective of this study was to test the effect of oestradiol-17 β (E_2) on motility, viability and acrosomal status of bull sperm *in vitro*.

Materials and Methods

Semen was obtained from two-year old Holstein bulls ($n = 3$) housed at a farm of the School of Agriculture, Selcuk University, Konya, Turkey. The semen was collected five times between 9:00 and 11:00 over a 9-mount time period, using an artificial vagina. Collected semen samples were pooled in a 50 mL centrifuge tube (C-8296, Sigma, Steinheim, Germany). The tube was brought to the laboratory within 20 min at 37 °C. Seminal plasma was removed by washing the semen with phosphate buffered saline (PBS, P5493, Sigma-Aldrich, Steinheim, Germany) containing 0.06 mg/mL penicillin-G (Potassium salt, P7794, Sigma-Aldrich, St Louis, USA). Washing was done by centrifuging twice at 1500 rpm for 5 min.

Oestradiol-17 β , 1.52 mg (E1024, Sigma-Aldrich, St Louis, USA), was dissolved in ethanol and the final concentration was adjusted to 0.4 $\mu\text{g}/\mu\text{L}$ with PBS. Four petri dishes (35 x 10 mm, P5112, Sigma-Aldrich, Steinheim, Germany) were prepared by adding 2.5 mL of PBS containing washed sperm cells. One petri dish without E_2 was kept as control (C). The other dishes were kept as test groups and 2 (T1), 4 (T2) and 8 (T3) $\mu\text{g } E_2/\text{mL}$ were added. After the addition of E_2 , the dishes were placed in an incubator at 18.5 °C for 24 h. Then, at 4, 18 and 24 h all the measurements were conducted by two different observers.

At the beginning of each experiment the number of motile sperm cells was counted, using a Makler counting chamber (Sefi-Medical, Haifa, Israel). Then at 4, 18 and 24 h of incubation, 2 μL of semen samples from each petri dish were transferred to a pre-warmed Makler counting chamber. The numbers of immotile, wriggling, rotating and forward moving sperm cells were counted under a light microscope at 200x magnification in 10 squares of the chamber. For each sample at last 200 sperm cells were counted at 5 different areas of the chamber and the percentages of motile sperm were calculated.

One gram Nigrosin (1.15924, Merck, Darmstadt, Germany) and 6.7 mL Eosin (Yellow, 45242, Fluka, Steinheim, Germany) was mixed. Then, 6 mL ultra pure water was added and dissolved for 20 min in a water bath at 100 °C and filtered. To the filtered stain, 0.68 mL of a glucose solution (100 g/L solution, G8644, Sigma-Aldrich, St Louis, USA) was added and rinsed with 6 mL of tartrate buffer. The stain was placed in a dark glass container and kept at 4 °C. A Nigrosin-Eosin (NE) and Giemsa mixed stain was prepared by mixing 900 μL of NE stain with 100 μL of Giemsa (Azur eosin methylene blue solution 1.09204, Merck, Darmstadt, Germany). To determine the initial count of morphologically abnormal sperm, slides ($n = 4$) were prepared and stained with NE and NE mixed with Giemsa.

For staining, 100 μL semen samples from each petri dish were placed in a test tube and fixed with 100 μL formaldehyde (FI/01FS/060929, Kimetsan, Turkey) for 5 min in a water bath at 37 °C. Then 100 μL of the NE stain or the 100 mixed stain (90 μL NE and 10 μL Giemsa) were added and left for 10 min at 37 °C. For each tube, two slides were prepared and counted at 100x magnification, using an oil immersion lens under a light microscope (Leica, DM 2500) equipped with a camera (Leica, DFC-280) connected to a computer. The acrosomal membranes were clearly seen (Figure 1).

At 0, 4, 18 and 24 h of incubation the number of live and dead sperm was counted on NE-stained slides using a light microscope under an oil immersion lens and a 100x magnification. Dead sperm cells were differentiated from live ones by their dark appearance. For each slide, 200 - 300 sperm were counted.

The numbers of all other morphologically abnormal sperm cells were counted using an oil immersion lens at 100x magnification under a light microscope connected to the video camera. On the computer screen the number of sperm possessing cytoplasmic droplets, coiled tails, lost and damaged acrosomes were counted at 0, 4, 18 and 24 h of the experiment on both N-E and N-E together with Giemsa stained slides.

Actual counts were analyzed by ANOVA (Randomized block design). Comparisons between individual treatments were tested using the least significant difference by an MSTAT-C statistical programme (Version 1.2., 1988, Michigan State University, East Lansing, MI 48824, USA) at a significance level of $P < 0.05$.

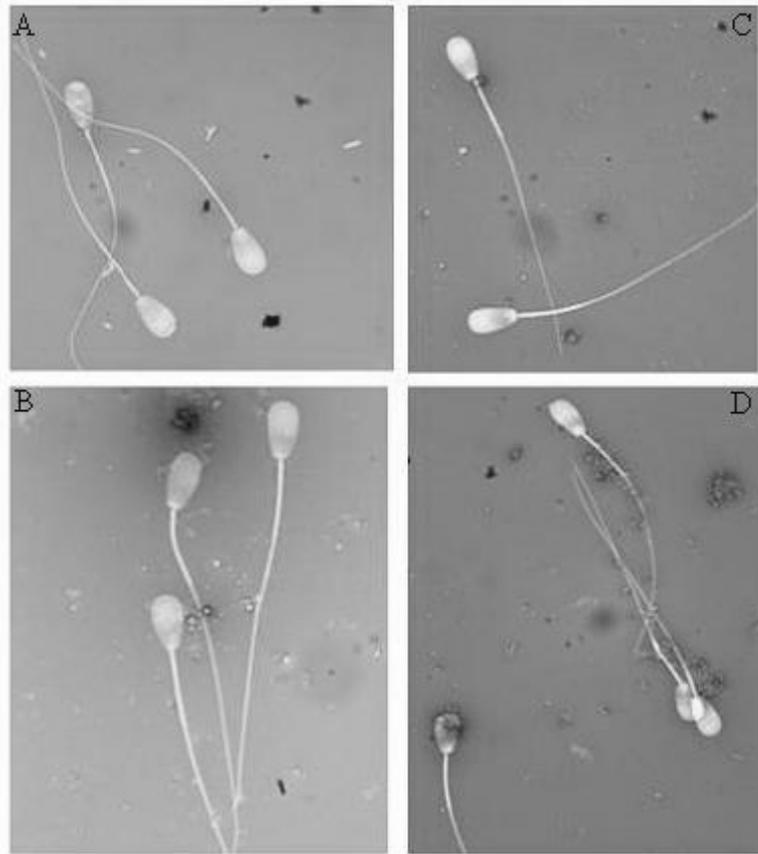


Figure 1 Acrosomal membranes stained with Nigrosin-Eosin (A and B) or Nigrosin-Eosin with Giemsa (C and D).

Results

Mean (\pm s.e.) percentages of motile sperm of bull semen incubated for 24 h in the presence of E_2 are displayed in Figure 2. After the addition of $2 \mu\text{g } E_2/\text{mL}$ at 18 h of incubation, the number of total motile sperm was increased as compared to the control group. Differences in total motility were not statistically important at other times. Figure 3 shows that the number of forward progressing sperm was significantly increased ($P < 0.05$) over the control group at the 4 h of incubation following the supplementation of 2 and $8 \mu\text{g } E_2/\text{mL}$. Mean (\pm s.e.) percentages of live and dead sperm displayed on Tables 1 and 2 indicate that lower doses of E_2 (2 and $4 \mu\text{g}/\text{mL}$) did not affect the number of dead and live sperm ($P > 0.05$), but a high dose E_2 ($8 \mu\text{g}/\text{mL}$) caused reduction ($P < 0.05$) of sperm quality at 4 and 24 h of incubation. Mean (\pm s.e.) percentages of sperm cells with a lost acrosome during 24 h of incubation displayed on Table 3 show that sperm cells with lost acrosomes were significantly higher ($P < 0.05$) in the control group at 24 h of incubation than in the other treatments. Number of sperm (%) bearing damaged acrosomes, coiled tail, proximal and distal cytoplasmic droplets during 24 h are shown in Table 4. It shows that oestradiol did not cause any damage ($P > 0.05$) to the acrosome. Also, the numbers of sperm possessing proximal and distal cytoplasmic droplets, and the numbers of sperm cells bearing coiled tails were not altered by any of the treatments.

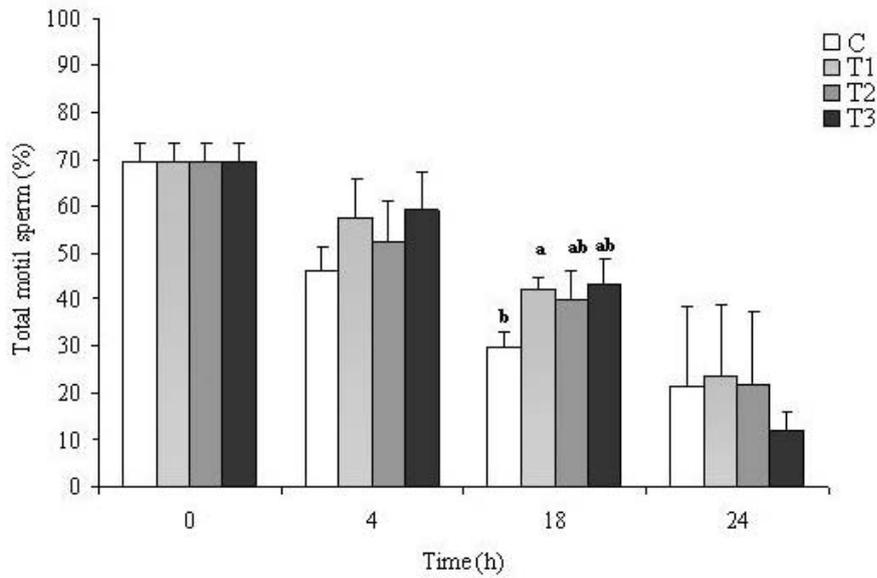


Figure 2 Mean (\pm s.e.) percentages motile sperm of bull semen incubated for 24 h at 18.5 °C with oestradiol-17 β (E_2). C is the control group while T1, T2 and T3 are treatment groups supplemented with 2 μ g, 4 μ g and 8 μ g E_2 /mL respectively.
^{a, b, ab} values with different superscripts differ significantly ($P < 0.05$).

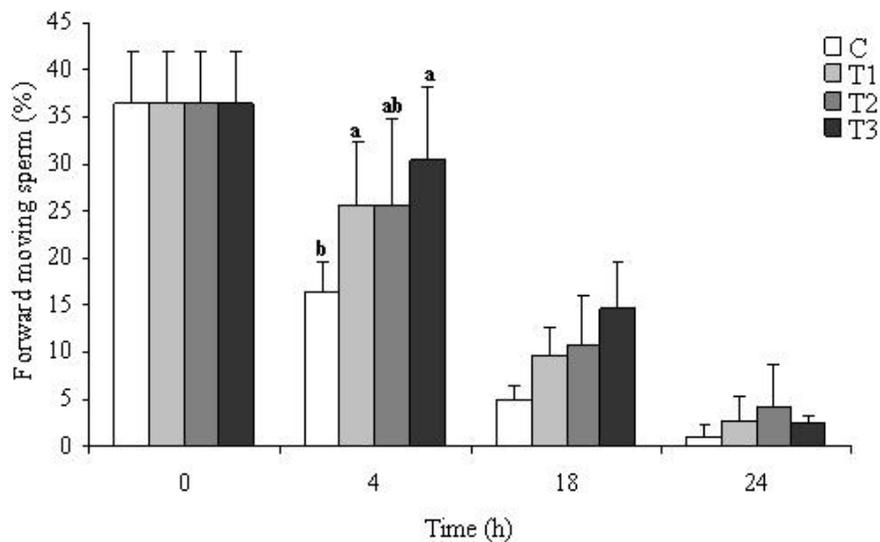


Figure 3 Percentage of forward moving sperm from pulled bull semen incubated with oestradiol-17 β (E_2) for 24 h at 18.5 °C. C is the control group while T1, T2 and T3 are the test groups supplemented with 2 μ g, 4 μ g and 8 μ g E_2 /mL, respectively.
^{a, b, ab} values with different superscripts over the columns differ significantly ($P < 0.05$).

Table 1 Mean (\pm s.e.) percentages of live sperm from pooled Holstein semen incubated at 18.5 °C for 24 h with oestradiol-17 β (E₂)

Groups	Percentage live sperm (%)			
	0 h	4 h	18 h	24 h
C	67.65 \pm 3.26	60.63 ^a \pm 5.74	48.14 \pm 1.76	39.27 ^a \pm 2.46
T1	67.65 \pm 3.26	59.03 ^{ab} \pm 4.25	46.40 \pm 4.33	40.92 ^a \pm 2.29
T2	67.65 \pm 3.26	56.79 ^{ab} \pm 5.72	46.02 \pm 3.05	42.61 ^a \pm 1.27
T3	67.65 \pm 3.26	53.11 ^{bc} \pm 5.31	49.41 \pm 0.46	31.69 ^b \pm 4.42

^{a, b, ab, bc} values with different superscripts within a column differ significantly (P < 0.05).

T1 - Test group-1, supplemented with 2 μ g E₂/mL;

T2 - Test group-2, supplemented with 4 μ g E₂/mL;

T3 - Test group-3, supplemented with 8 μ g E₂/mL.

Table 2 Mean (\pm s.e.) percentage of dead sperm of pooled Holstein semen incubated at 18.5 °C for 24 h with oestradiol-17 β (E₂)

Treatments	Percentage of dead sperm (%)			
	0 h	4 h	18 h	24 h
C	32.35 \pm 3.26	39.37 ^a \pm 5.74	51.86 \pm 1.76	60.73 ^a \pm 2.46
T1	32.35 \pm 3.26	40.97 ^{ab} \pm 4.25	53.60 \pm 4.33	58.08 ^a \pm 2.29
T2	32.35 \pm 3.26	43.21 ^{ab} \pm 5.72	53.98 \pm 3.05	57.39 ^a \pm 1.27
T3	32.35 \pm 3.26	46.73 ^{bc} \pm 5.31	50.59 \pm 0.46	68.31 ^b \pm 4.42

^{a, b, ab, bc} values with different superscripts within a column differ significantly (P < 0.05).

T1 - Test group-1, supplemented with 2 μ g E₂/mL;

T2 - Test group-2, supplemented with 4 μ g E₂/mL;

T3 - Test group-3, supplemented with 8 μ g E₂/mL.

Table 3 Mean (\pm s.e.) percentage sperm cells with lost acrosomes during 24 h of incubation at 18.5 °C. Data are displayed as mean and standard error

Treatments	Number of sperm with lost acrosomes (%)			
	0 h	4 h	18 h	24 h
C	7.52 \pm 1.14	9.06 \pm 0.94	8.88 \pm 0.35	14.25 ^a \pm 2.57
T1	7.52 \pm 1.14	7.64 \pm 0.94	8.38 \pm 0.30	10.51 ^b \pm 1.85
T2	7.52 \pm 1.14	10.91 \pm 0.83	10.84 \pm 1.53	10.12 ^b \pm 2.06
T3	7.52 \pm 1.14	10.02 \pm 2.19	9.06 \pm 1.16	11.43 ^{ab} \pm 2.07

^{a, b, ab} values with different superscripts within a column differ significantly (P < 0.05).

Semen, from three bulls, collected five times (n = 5). C is control group, T1, T2 and T3 are the test groups supplemented with 2 μ g, 4 μ g and 8 μ g oestradiol-17 β (E₂), respectively.

Discussion

Upon the addition of 2 μ g E₂/mL, total motility increased at 18 h of incubation, but was not affected by adding 4 or 8 μ g E₂/mL. This increase in total motility might be a result of the increase in the number of

forward progressing sperm cells at 4 h of incubation. These forward progressing sperm cells at 4 h were rotating and wriggling at 18 h of incubation. Here a positive correlation was observed between E₂ supplementation and the number motile sperm. A similar positive correlation was reported by Devkota *et al.* (2008) in post-pubertal Holstein bulls. They investigated the correlation between peripheral E₂ concentration and sperm motility *in vivo*. They reported that sperm motility after collection and after freezing and thawing was correlated positively with the E₂ concentration. According to another study, pre-treatment of bovine sperm cells with E₂ or oestrous stage-specific serum resulted in a higher percentage of motile sperm after 18 h in isthmic and after 36 h in ampullary cultures compared with the control (Boquest & Summers, 1999). They also concluded that during the pre-ovulatory period when the dominating hormone is E₂, the bovine oviduct provides an optimal environment for the survival of sperm.

Table 4 Number of sperm (%) bearing damaged acrosomes, coiled tails, proximal and distal cytoplasmic droplets during 24 h incubation at 18.5 °C with oestradiol-17 β (E₂)

Abnormalities (%)	Treatments	Time (h)			
		0	4	18	24
Damaged acrosomes	C	5.36 ± 0.99	7.54 ± 1.42	6.74 ± 0.49	10.55 ± 1.4
	T1	5.36 ± 0.99	6.26 ± 1.39	6.14 ± 0.62	12.41 ± 3.01
	T2	5.36 ± 0.99	8.92 ± 1.10	7.19 ± 0.57	11.26 ± 2.91
	T3	5.36 ± 0.99	9.08 ± 1.24	6.42 ± 0.34	9.56 ± 2.61
Proximal cytoplasmic droplets	C	2.35 ± 1.36	1.07 ± 0.34	0.65 ± 0.17	0.55 ± 0.23
	T1	2.35 ± 1.36	1.53 ± 0.41	0.64 ± 0.33	0.44 ± 0.23
	T2	2.35 ± 1.36	0.61 ± 0.29	0.50 ± 0.36	0.47 ± 0.32
	T3	2.35 ± 1.36	0.61 ± 0.19	0.41 ± 0.10	0.50 ± 0.12
Distal cytoplasmic droplets	C	2.18 ± 0.50	2.13 ± 1.20	0.32 ± 0.22	0.63 ± 0.23
	T1	2.18 ± 0.50	1.13 ± 0.52	0.89 ± 0.26	1.07 ± 0.76
	T2	2.18 ± 0.50	1.58 ± 0.47	0.61 ± 0.30	1.12 ± 0.61
	T3	2.18 ± 0.50	0.95 ± 0.32	0.39 ± 0.26	0.58 ± 0.18
Coiled tails	C	1.86 ± 0.98	0.95 ± 0.37	1.79 ± 1.19	0.57 ± 0.25
	T1	1.86 ± 0.98	0.62 ± 0.27	0.73 ± 0.34	0.38 ± 0.20
	T2	1.86 ± 0.98	0.74 ± 0.25	1.41 ± 0.93	0.35 ± 0.20
	T3	1.86 ± 0.98	0.71 ± 0.30	1.32 ± 0.82	0.31 ± 0.09

Differences are not statistically significant (P >0.05). C is control group, T1, T2 and T3: are the test groups supplemented with 2 μ g, 4 μ g and 8 μ g E₂ / mL respectively.

Similar results to the present study have been reported by Jin *et al.* (2005), where implanting male hamster with silastic tubes containing either a low or high amount of E₂ increased sperm motility. The physiological reason behind increased forward motility due to the E₂ addition is not presently known. One possibility might be the involvement of cAMP signal transduction pathways. It has been reported that these pathways are involved in activation of sperm motility (Armstrong *et al.*, 1994; Wade *et al.*, 2003). This increase in motility is an important factor affecting fertilization, because high rates of ovum fertilization require efficient transport of sperm from the cervix to the oviducts (Hawk *et al.*, 1978). According to a number of studies, when a specific number of motile sperm cells were added to oocytes, an equal number of oocytes was fertilized (First & Parrish, 1987; Purdy & Graham, 2004).

In this study sperm viability was not affected by the supplementation of 2 and 4 μ g E₂/mL, but it was significantly decreased by the addition of 8 μ g E₂/mL. Higher doses of E₂ are detrimental for sperm survival. A study was conducted by Lukoseviciute *et al.* (2005) to determine the effect of 1 μ g E₂/mL on sperm plasma membrane scrambling, capacitation and acrosome reaction of post-thaw bovine sperm. According to the result of their study, the addition of 1 μ g E₂/mL did not affect sperm viability. In this study even 2 μ g

E₂/mL did not affect sperm viability. Therefore, lower doses of E₂ do not alter sperm survival, but higher doses are detrimental.

According to our results, the number of sperm with lost acrosomes is not significantly ($P > 0.05$) affected by E₂ supplementation during 18 h of incubation, while the number of sperm with lost acrosomes was significantly lower ($P < 0.05$) in test groups containing 2 and 4 µg E₂/mL compared to the control group at 24 h of incubation. In the control group, the higher number of sperm with lost acrosomes indicates the presence of a significantly higher number of acrosome-reacted sperm. The addition of E₂ promoted the sperm to keep acrosomes at an intact state which is necessary for binding of sperm to the zona pellucida, for subsequent fertilization. In the control group the high number of sperm cells with lost acrosomes is not desirable, since sperm with lost acrosomes will no longer be able to interact with an unfertilized oocyte for successful fertilization (Yanagimachi, 1994).

In this study, addition of E₂ to bull sperm did not cause any significant differences in the number of sperm cells with cytoplasmic droplets. But according to an *in vivo* study conducted on adult male mice by Cho *et al.* (2003), a positive effect of oestrogen on sperm maturation in the epididymis was suggested. Our results are different and may be attributed to species difference.

Conclusions

A lower dose (2 µg/mL) E₂ has a beneficial effect on the motility and the acrosomal integrity of bull sperm *in vitro*. Sperm survival was not affected by the supplementation of lower doses (2 and 4 µg/mL) E₂ while a high dose (8 µg/mL) of E₂ was detrimental for sperm survival at 24 h of the incubation.

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