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SOME PHYSIOLOGICAL EFFECTS OF STEROID HORMONES ON SEMEN CHARACTERISTICS OF AWASSI AND BARKI RAMS

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ABSTRACT

The aim of this study was to investigate the effects of addition of two steroid hormones, namely progesterone and testosterone, to the extender of ram semen on the quality, maintenance and storageability of pelleted rams spermatozoa. The effects of different incubation periods, at 37°C were also studied. Two successive semen ejaculates were collected twice weekly for 16 weeks from eight sexually mature rams (4 barki and 4 Awassi).

Results showed that breed of rams have no significant effects on sperm motility, acrosome reaction (AR) or percentage of dead spermatozoa. Progesterone had on inhibiting significant effects on sperm motility whether added to the extender at pre-cooling or post-thawing. Adding testosterone at pre-cooling or post-thawing maintained sperm motility. Acrosomal reaction was affected significantly by adding either progesterone or testosterone whether at pre-cooling or post-thawing as compared to control. Percentage of dead spermatozoa significantly increased by addition of progesterone at post-thawing and testosterone at pre-cooling as compared to control while adding testosterone at post-thawing was significantly decrease the percentage of dead spermatozoa in ram sperm. Sperm motility decreased significantly by using progesterone or testosterone addition at pre-cooling or post-thawing as incubation at 37°C advanced. Adding testosterone or progesterone at pre-cooling or post-thawing did not drastically affect acrosome reaction, but differences were significant as compared to control. Dead spermatozoa were also significantly increased by the different treatments compared to control.

INTRODUCTION

Successful artificial insemination is definitely dependent on conservation and banking of frozen semen. The greatest problem however, in sperm preservation is the low recovery index manifested in the depression of post-thawing motility as compared to fresh sperm motility (Richter *et al.*, 1984). Crystallization and respiration shock occurring post-thawing cause 25% decreases in fertilizing power (Behman and Sawada, 1966). With this problem in mind, several studies have been carried out using the stimulatory effect of some steroid hormones on fresh sperm functions such as progesterone (Nemere and Carson, 1998; Luconi *et al.*, 1999) and testosterone (Ailenberg *et al.*, 1984 and Salem *et al.*, 1990). Several studies have demonstrated that serum in the high concentration of progesterone can elicit recognizable changes in the pattern of sperm movement (Mbizvo *et al.*, 1990) as wall as increases in the acrosome reaction (Osman *et al.*, 1989). It is generally accepted that sperm motility and the type of this motility influence the fertilizing ability of sperm.

The effect of steroids, in particular on sperm motility, is unknown and their postulated effects in sperm membranes totally hypothetical (Blasco and Wolf, 1977). The acrosome is required for penetration of the zona pellucida, an extracellular glycoprotein egg envelope, and for sperm-egg plasma membrane fusion in etherian mammals (Bedford, 1998 and Yanagimachi,

1994). Also, despite a great deal of research, the signal transduction pathways involved in the progesterone-initiated mammalian sperm AR are not yet fully understood (Harrison, 2000).

The role of these hormones seems to be directed towards controlling specific processes such as motility and acrosome reaction (Rolden *et al.*, 1994). The mechanism of progesterone action is most probably mediated by progesterone receptor resident in the plasma membrane of sperm. Binding of progesterone to this receptor then activates the Ca^{++}-channel in the plasma membrane or inhibits the plasma membrane Ca^{++} ATPase pump. Alternatively the progesterone receptor may have inherent Ca^{++}-channel activity it self (Ghobashi and Anwar, 2004). Mendoza *et al.* (1995), and Tesarik and Mendoza (1996) suggested at least three receptors by mean of which progesterone targets, respectively, a plasma membrane calcium channel, a plasma membrane chloride channel and a membrane-associated protein tyrosine kinas. The later of these three receptors seems to be that visualized by the hormone-binding assay because those spermatozoa that bind the conjugate also selectively increase their phosphotyrosine content and initiate the acrosome reaction (Kholkute *et al.*, 1995).

As early as 1972, Wester and Foote indicated that testosterone stimulates bovine sperm motility. This was also found by Voglmyr *et al.* (1970) and Voglmyr (1971) who suggested that adding testosterone to ejaculated bovine spermatozoa decreased oxygen consumption but increased the rate of aerobic glycolysis. Also, Beck *et al.* (1976) found that incubation of 5 ng/ml testosterone (as physiological level) caused acceleration of spermatozoa. The effect of testosterone on enhanced motility may be attributed to its positive effects on the rate of glycolysis and hence on increasing energy production.

MATERIALS AND METHODS

Animals and semen collection :

This study was carried out at the Alexandria University Experimental Station and Borg El-Arab Research Station under the Research Project No. 17 which funded by the Ministry of the Agriculture and Land Reclamation (MLR). Semen samples were collected from eight rams (4 Awassi and 4 Barki) of 2-3 years of age. Two ejaculates were collected from each ram twice per week by artificial vagina. The semen samples were transferred immediately after collection to the laboratory where it was placed in a water bath (37°C) until used. Semen ejaculated were initially examined for volume, color, progressive motility (Perry, 1960), percentage of dead spermatozoa (Blom, 1950 and Dott, 1956) and acrosomal reaction (Bryan and Akruk, 1977 and modified by Lenz *et al.*, 1983). Ejaculates (not less than 70% motility) and minimum percentages of dead and abnormal spermatozoa were used. Throughout this study selected ejaculates within each of the two breeds under investigation were pooled together to get the needed volume for further processing.

Preparation of used materials :

Tris extender recommended by El-Bhrawi (2002) was used throughout the present experiment. This extender is a primary amine buffer with a molecular weight of 121.14 and a buffering capacity of pH 7.0 to pH 9.0

$(CH_2OH)_3CNH_2$) and progesterone (4 pregnene 3, 20 dione) and testosterone (17 □-hydroxy-4-androsten-3-one) hormones were dissolved in ethanol and then diluted with a buffer solution to get the required concentrations. Levels of testosterone and progesterone used were 40 □g/ml and 20 □g/ml, respectively.

Tris extender (37°C) was used to dilute all pooled semen samples at a rate of 1 semen sample to 8 extender in a glass tubes. This might provide about 80.4 × 10^6 motile sperm 0.2 ml pellet before freezing (pilot experiment). Each pooled ejaculate was distributed among all treatments assigned in an experiment, so each pooled ejaculated was represented in all treatment under investigation. Tubes were covered with parafilm and placed in the refrigerator at 5°C for 4-6 hours for gradual cooling and equilibration, after that tubes containing the extended semen were then placed in container filled with cooled water to hold the temperature of the extended semen at 5°C. Small depressions were made on a block of solid carbon dioxide (dry ice- 79°C) to accommodate the droplets of semen (0.2 ml) for freezing. Droplets of extender semen were dispensed by the use of 0.2 ml automatic micro-dispenser (Sodipe, 1989).

Sub-samples were taken from the cooled semen for motility evaluation at different times post-cooling. Concerning post-thawing motility, frozen semen pellets were thawed in 1 ml of 2.9% sodium citrate dehydrate at 40°C for 30 sec. Thawed semen was mixed before sampling for microscopic evaluation of sperm motility. Post-thawing motility evaluation was performed immediately post-thawing (0 hour) and at 1, 2 and 4 hours post-incubation at 37°C.

Experimental design :

Pooled seminal ejaculates of each ram of each breed were splited into three parts. The first part was used as a control while the second part split into two portions. Testosterone or progesterone was added to one portion before freezing (PRC). The two hormones were added to the third portion at post-thawing (PTT).

Percentage of sperm motility (%), AR and dead spermatozoa (%) were assessed in all samples after cooling and post-thawing and at incubation (37°C) for 0,1, 2 and 4 hours.

Statistical analysis :

Least square analysis of variance using General Linear Models (GLM) procedure was performed (Statistix, 2000). Significant differences between means were detected using Duncan's multiple range tests (Duncan, 1966).

RESULTS AND DISCUSSION

The data in Table (1) shows no statistical significant difference between the two breeds of rams under study in sperm motility, AR and dead spermatozoa percentages. It was also found that added testosterone at post-thawing significantly ($P<0.05$) increased motility percentage than pre-cooling addition (Table 2). On the other hand, addition of progesterone at pre-cooling or post-thawing lowered significantly ($P<0.05$) sperm motility as compared to control, and both were significantly ($P<0.05$) lower than that of pre-cooling or post-thawing in the addition of testosterone (Table 2). However, motility percentage resulted after post-thawing in the addition of progesterone did not

significantly differ than that of pre-cooling (Table 2). The effect of progesterone in sperm motility capacitation and acrosome reaction is partially reproduced by the □-amino-butiric acid (GABA) receptor antagonist picrotoxin. A GABA-like receptor has been shown to be involved in progesterone-induced acrosome reaction (Shi and Roldan, 1995), but not in the progesterone-induced Ca^{2+} influx (Blackmore and Lattanzio, 1991 and Turner *et al.*, 1994). This could explain why GABA does not completely reproduce and picrotoxin doses not totally abolish the effect of progesterone on cAMP levels.

Table (1): Breed differences in percentages of sperm motility, acrosome reaction (AR) and dead spermatozoa

Parameters		Breed	
		Barki	Awassi
Motility	(%)	62.7 ±0.82	64.6 ±0.29
AR	(%)	15.3 ±1.40	14.7 ±0.49
Dead	(%)	27.7±6.05	26.7 ±0.23

The effect of testosterone in stimulating bovine sperm motility was also documented by Wester and Foote (1972), Beck (1976), and Beck *et al.* (1976). Voglmyr *et al.* (1970) and Voglmyr (1971) reported that testosterone may increase the rate of sperm cell glucolysis and interne increase energy production of the sperm resulted in increased motility. While, Salem *et al.* (1990) reported that ejaculated washed ram sperm showed consisted increase in the intracellular concentration of cyclic AMP following incubation for 15 min with graded doses (15, 30 or 60 µg/ml) of testosterone, dihydro-testosterone (DHT) or androstenedione. These doses of androgens significantly enhanced the rate of protein and RNA synthesis by the washed ram sperm during a 4 h incubation period. The addition of testosterone or DHT to ram semen *in vitro* also stimulated and maintained sperm motility and enhanced the rate of fructose utilization. These results indicated that androgens could play important roles in the metabolism of mature sperm. Androgens (possible mediated by cAMP) seem to be involved in *de nove* synthesis of proteins (enzymes) that stimulate sperm fructolysis and provide energy for sperm motility.

Table (2): Overall effects of testosterone and progesterone addition at pre-cooling (PRC) and post-thawing (PTT) on percentages of motility, acrosome reaction (AR) and dead spermatozoa of rams at pre-cooling and post-thawing

Treatment	Parameters		
	Motility (%)	AR (%)	Dead (%)
Control*	68.7^{a}	13.0^{a}	21.5^{b}
Testosterone			
PRC	65.9^{b}	12.6^{b}	23.0^{b}
PTT	69.1^{a}	11.3^{c}	18.2^{c}
Progesterone			
PRC	61.2^{c}	10.8^{c}	22.2^{ab}
PTT	60.6^{c}	12.3^{b}	23.8^{a}

* Control : Raw semen with extender only.

a-c : Values with different superscripts within columns differ significantly ($P<0.05$).

Allenberg *et al.* (1984) reported that testosterone inhibited collectively motility of ejaculated ram spermatozoa in a dose-dependent fashion. The effect of testosterone was noted both on cells suspended in buffer containing fructose and exogenously starved cells treated with fructose after collective motility arrest. It was concluded that testosterone might exert its inhibitory effect on motility by uncoupling fructolytic energy production to the tail motility system.

Results indicated that adding either testosterone or progesterone at pre-cooling or post-thawing had significant lower effects on spermatozoa AR as compared to control (Table 2). Also, it's indicated that adding either testosterone or progesterone caused statistically significant difference ($P<0.05$) between the pre-cooling and post-thawing (Table 2). It was also found that adding progesterone post-thawing significantly ($P<0.05$) increased dead spermatozoa percentage as compared to control and at adding testosterone post-thawing although it had no effects on adding testosterone or progesterone pre-cooling (Table 3). From these data it can be concluded that the addition of testosterone or progesterone to semen samples post-thawing (PTT) increase the quality of semen as it increase significantly ($P<0.05$) sperm motility also it had statistically significant difference ($P<0.05$) on AR or dead spermatozoa.

Values of sperm motility, AR and dead spermatozoa of adding either testosterone or progesterone at pre-cooling or incubated for different times 0, 1, 2 and 4 hrs at 37°C (post-thawing) are shown in Table (3). Results indicated that adding testosterone at pre-cooling resulted a significant higher ($P<0.05$) sperm motility and AR percentage (Table 3) at pre-cooling than those incubated for different period at 37°C.

Adding testosterone at pre-cooling resulted lowest significant ($P<0.05$) dead spermatozoa than adding testosterone pre-cooling and incubated for 4 hr at 37°C. On the other hand, adding progesterone at pre-cooling increased significantly ($P<0.05$) sperm motility and AR percentage than those incubated for different periods. Results also indicated that adding testosterone at post-thawing significantly ($P<0.05$) increased sperm motility as compared to the pre-cooling addition or pre-cooling. Although the addition of the two additives almost has the same trend, testosterone seems to have a more pronounced effect on motility than progesterone. As early as 1972, Wester and Foote indicated that testosterone stimulates bovine sperm motility. Maximal stimulation was found with 10 ng testosterone per 25×10^8 spermatozoa per milliliter. Also, Beck *et al.* (1976) reported that incubation 5 ng/ml testosterone (as a physiological level) caused acceleration of human sperm motility. The effect of testosterone on enhanced motility may be attributed to its positive effects on the rate of glycolysis and hence on increasing energy production. On the other hand, adding progesterone at post thawing significantly ($P<0.05$) increased sperm motility as compared to the pre-cooling addition without any addition except incubation period (2 or 4 hrs). The prsent results indicated that adding testosterone at post-thawing significantly ($P<0.05$) decreased AR percentage as compared to the incubation period (1, 2 and 4 hr). Data in Table (3) show that adding testosterone in pre-cooling or post-thawing have the same trend. Results indicated that adding progesterone at post-thawing increased AR percentage as compared to the pre-cooling. The effect of progesterone on increasing AR after incubation had been demonstrated by Barboni *et al.* (1995). They reported that *pisum sativum*

agglutinin (FITc-PsA) staining showed that progesterone did not affect the incidence of spontaneous AR. By contrast, spermatozoa incubated with progesterone showed a higher percentage of AR. Also, Ghobashy and Anwar (2004) found that this effect probably mediated by specific receptor on plasma membrane of sperm which activates the Ca^{2++} influence through plasma membrane throughout inhibits on the plasma membrane Ca^{2+}-ATPase pump. Percentages of dead spermatozoa were increased as affected by progress period of incubation time at 37°C in pre-cooling and post-thawing treatments when testosterone or progesterone was added.

Generally, we found that dead spermatozoa increased significantly ($P<0.05$) by adding testosterone at pre-cooling as compared to post-thawing but adding progesterone increased in post-thawing at different incubated periods than pre-cooling.

Table (3): Overall mean effects of addition testosterone and progesterone at pre-cooling (PRC) and during incubation period post-thawing (PTT) at 37°C on the percentage of sperm motility, acrosome reaction (AR) and dead spermatozoa

	control	Pre-cooling	Incubation period (hrs) (PTT)			
			0	1	2	4
(%)	Testosterone (PRC)					
Motility	75.3^{a}	66.7^{b}	63.3^{c}	60.7^{d}	56.7^{e}	51.7^{f}
AR	79.3^{a}	69.2^{b}	68.0^{b}	62.5^{c}	60.0^{d}	55.5^{e}
Dead	18.5^{e}	23.5^{d}	28.7^{c}	31.3^{b}	35.5^{ab}	36.5^{a}
(%)	Progesterone (PRC)					
Motility	75.8^{a}	73.5^{b}	59.1^{c}	53.5^{d}	47.1^{e}	40.0^{f}
AR	75.2^{a}	71.6^{b}	66.7^{c}	61.3^{d}	55.9^{e}	45.9^{f}
Dead	20.0^{f}	22.5^{e}	28.5^{d}	32.2^{c}	39.9^{b}	50.3^{a}
(%)	Testosterone (PTT)					
Motility	81.7^{a}	71.7^{b}	66.7^{c}	61.7^{d}	59.7^{e}	53.3^{f}
AR	79.7^{a}	78.5^{b}	76.7^{c}	66.2^{d}	54.7^{e}	46.2^{f}
Dead	17.7^{f}	20.7^{e}	23.8^{d}	28.3^{c}	32.8^{b}	46.0^{a}
(%)	Progesterone (PTT)					
Motility	81.7^{a}	77.0^{b}	62.9^{c}	55.0^{d}	46.8^{e}	35.0^{f}
AR	78.0^{a}	77.5^{a}	73.2^{b}	64.5^{c}	57.8^{d}	52.0^{e}
Dead	16.5^{f}	22.5^{e}	30.1^{d}	34.7^{c}	42.4^{b}	54.5^{a}

a-f: Values of each criteria with different superscripts within different raws differ significantly ($P<0.05$)

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بعض التأثيرات الفسيولوجية للهرمونات الإستيرويدية على خواص السائل المنوي لذكور أغنام العواسي والبرقي

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كان الهدف من البحث هو دراسة تأثير إضافة اثنين من الهرمونات الإستيرويدية هما البروجستيرون والتستستيرون على جودة السائل المنوي لكباش العواسي والبرقي، وإمكانية المحافظة على الوظائف الحيوية (النسبة المئوية لمعدل الحركة التقدمية والتهيئة للإخصاب والحيوانات المنوية الحية والميتة) لهذا السائل عند حفظه بالتجميد في صورة حبيبات.

تم جمع السائل المنوي مرتين أسبوعياً لمدة ١٦ أسبوع من ٨ ذكور أغنام بالغة (٤ عواسي، ٤ برقي) وقد أشارت النتائج إلى عدم وجود تأثير للسلالة على الوظائف الحيوية للسائل المنوي.

البروجيستيرون كان له تأثير مثبط على الحركة التقدمية للسبرمات سواء أضيف قبل التبريد أو بعد الإسالة على عكس التستستيرون الذي حافظ على هذه النسبة عند إضافته بعد الإسالة بينما انخفضت النسبة انخفاضاً معنوياً عند إضافته قبل التبريد. تأثر معدل التهيؤ للإخصاب معنوياً بإضافة أي من الهرمونين سواء أضيف قبل التبريد أو بعد الإسالة بينما زادت معنوياً النسبة المئوية للإسبرمات الميتة عند إضافة البروجستيرون بعد الإسالة وعند إضافة التستستيرون قبل التبريد.

عند مقارنة النتائج اثناء التحضين على ٣٧°م لمدة ١، ٢، ٤ ساعة وجد ان اضافة أي من الهرمونين سواء قبل التبريد او بعد الاسالة قد أدى لحدوث انخفاض معنوى فى النسبة المئوية لمعدل الحركة التقدمية للاسبرمات بينما لم يكن هناك تاثير شديد على النسبة المئوية لمعدل التهيؤ للإخصاب على الرغم من انخفاضه معنويا مقارنة بالكنترول. زادت النسبة المئوية للإسبرمات الميتة معنويا بالمعاملات المختلفة وعند التحضين لازمنة مختلفة مقارنة بالكنترول.