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EFFECT OF DIFFERENT TYPES AND LEVELS OF ANTIOXIDANTS ON VIABILITY AND ACROSOMAL STATUS OF FROZEN-THAWED SPERMATOZOA OF BUFFALO BULLS.

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ABSTRACT

Semen was collected twice daily (100 ejaculates) from 5 sexually mature buffalo bulls (7-10 y old and 550-620 kg LBW) during the interval from January to April 2007 to study the effect of different types and levels of antioxidants on sperm motility and acrosome status in post-thawed buffalo semen. The main extender used for semen dilution was Tris-egg-yolk-citrate containing 7% glycerol. Total of 10 extenders, 9 with 3 types and 3 levels of antioxidants, including catalase (250, 500 and 1000 IU), glutathione (GSH, 0.4, 0.8 and 1.2 mM) and ascorbic acid (AA, 0.5, 1.0 and 1.5 g/l) were compared to unsupplemented extender (control). Semen was extended with different extenders, frozen in Liquid nitrogen (-196^{o}C) and thawed at rates of 15/60, 35/30 and 55/15 ^{o}C/sec. Percentages of sperm motility and damage acrosome were determined in post-thawed semen. Conception rate was detected in 100 sexually mature buffalo cows. Results revealed that adding all antioxidants to semen extenders increased percentage of sperm motility compared with the control, except for the highest level of AA (1.5 g/l), which did not affect sperm motility percentage. The best improvement was catalase at a level of 1000 IU (62.1%). Adding all levels of catalase and the lowest level of GSH (0.4 mM) or AA (0.5 g/l) decreased percentage of sperm with damage acrosome as compared to the control. The highest level of catalase (1000 IU) showed the highest impact on reducing percentage of sperm with damage acrosome (6.0%). Sperm motility percentage was higher with a thawed rate of 55/15 than 35/30 than 15^{o}C/60 sec, being 43.0, 32.11 and 27.0%, respectively). Adding catalase (1000 IU) or AA (0.5 or 1.0 g/l) increased sperm motility percentage in semen thawed by different rates, being 38.3, 37.4 and 37.1%, respectively. The highest conception rate (80%) was obtained from buffalo cows inseminated with semen extended using catalase (1000 IU). In conclusion, Tris-based extender containing catalase at a level of 1000 IU in frozen semen thawed at a rate of 55^{o}C/15 sec showed the highest post-thawing motility and the best fertilizing capacity of buffalo spermatozoa.

Keywords: Egyptian buffaloes, freezing, thawing, sperm motility, acrosome, antioxidants.

INTRODUCTION

During the freezing process, the increase in susceptibility of spermatozoa to lipid peroxidation, as affected by cold shock, plays an important role in ageing of spermatozoa, shortening their life span and affecting the preservation of semen. Buffalo spermatozoa contain comparatively more unsaturated fatty acids than in other species, like

arachidonic and decosahexaenoic acids, which make them more vulnerable to lipid peroxidation (Singh *et al.*, 1989 and Sreejith *et al.*, 2005).

The cytoplasm of somatic cells contains several antioxidant enzyme systems, catalase, glutathione (GSH) and superoxide dismutase (antioxidants enzymes active in scavenging ROS). However, sperm cells are devoid of most of this cytoplasm, so the antioxidant system in sperm cells of different species is weak (Li, 1975). Semen contains appreciable amounts of antioxidants that balance lipid peroxidation and prevent excessive peroxide formation (Lewis *et al.*, 1997).

However, the endogenous antioxidative capacity of semen may be insufficient during storage or dilution (Maxwell and Salmon, 1993). In vitro studies suggested that the addition of an antioxidant (GSH) to diluted semen could improve the motility and survival of bull spermatozoa in frozen semen (El-Nenaey *et al.*, 2006). Recently, Ahmed (2008) found that addition of antioxidants (GSH or ascorbic acid) to extender of frozen buffalo semen improved sperm characteristics.

Sperm cells used in AI are exposed to oxygen and visible light radiation during various processing procedure or in semen stored by cooling or at room temperature, which could lead to formation of ROS, and negatively affect sperm cell motility and genomic integrity (Aitkin and Clarkson, 1988; Storey, 1997; Aitkin *et al.*, 1998 and Bilodeau *et al.*, 2001). Under these conditions, adding several types of antioxidants could help to maintain survival and motility of spermatozoa (Bilodeau *et al.*, 2001 and Foote *et al.*, 2002).

Therefore, the current work aimed to study the effect of adding different types and levels of antioxidants (catalase at levels of 250, 500 and 1000 IU, glutathione (GSH) at levels of 0.4, 0.8 and 1.2 mM or ascorbic acid (AA) at levels of 0.5, 1.0 and 1.5 g/l to Tris-egg yolk-citrate extender of frozen semen) on sperm motility and acrosome status in post-thawed buffalo semen thawed at different thawing rates.

MATERIALS AND METHODS

This study was carried out at Department of Animal Production, Faculty of Agriculture, Mansoura University and the experimental work was conducted at El-Gemmezah Experimental Station, belonging to Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt during the period from January to April 2007.

Animals and management system:

Five sexually mature buffalo bulls aged 7-10 years and weighed 550-620 kg were used for semen collection. Buffalo bulls were fed formulated diets on the basis of recommendation of APRI for adult buffalo's bull requirements. All bulls were fed daily ration composed of 8 kg concentrate feed mixture (CFM), fresh berseem (14 kg) and rice straw (4 kg). The ration was given individually to all bulls at 8.0 a.m. and 3.0 p.m., while, fresh water and mineral blocks were available for all bulls at all day times.

The CFM was composed of 32% undecorticated cotton seed cake, 26% wheat bran, 22% yellow maize, 12% rice bran, 5% linseed meal, 2%

vines, 0.5% limestone and 0.5% NaCl. Animals were housed individually under semi-open sheds.

Semen collection:

Semen was collected during the interval from January to April 2007 by means of an artificial vagina set up at optimal conditions to induce a good ejaculatory thrust. At the time of collection, a buffalo bull was used as a teaser. One false mount had been allowed before collection of the first ejaculates. Ejaculates were obtained from each buffalo bull twice/week early in the morning (7 a.m.) for 10 collection weeks (100 ejaculates).

Immediately after semen collection, the ejaculates were transferred to the laboratory and were placed in a water bath at 37ºC and care was taken to avoid exposure of the semen to any unfavorable conditions during or after collection. Ejaculates taken from all bulls (only with ≥70% sperm mass motility) on each collection day were pooled and divided into 9 portions for dilution.

Semen dilution and treatments:

The main extender used for semen dilution was TEYC extender containing 7% glycerol. Total of 10 extenders, 9 of them with 3 types of antioxidant with three levels including catalase (250, 500 and 1000 IU), glutathione (GSH, 0.4, 0.8 and 1.2 mM) and ascorbic acid (AA, 0.5, 1.0 and 1.5 g/l) were compared to extender with no additive (control).

Semen was extended with different types and levels of antioxidant at a rate of 1 semen: 8 extender at 37ºC, and cooled to 5°C for 6 hours as equilibration period. Semen was frozen in straws (500 straws, 50 ones for each). All semen straws were thawed at a rate of 37°C/30 sec.

Additional semen straws (with different types and level of antioxidant was evaluated with three thawing rate including 15 ºC for 60 sec, 35 ºC for 30 sec and 55 ºC for 15 sec.

Freezing processes:

After semen dilution, the vial containing the extended semen were placed in a water bath at 37°C and then placed into refrigerator at 5°C for 6 hours. For gradual cooling, straws were kept in iced water bath to keep its temperature at 5°C, while semen packed in straws was placed in a cooled ice chest.

The extended cooled semen equilibrated for 6 hours was transferred into processing container and located horizontally in static nitrogen vapor 4 cm above the surface of liquid nitrogen for 10 minutes, then the straws were placed vertically in a metal canister and immersed completely in liquid nitrogen container for storage at -196°C for one month. Each straw was titled for each treatment and collection week.

Semen evaluation:

Percentages of sperm motility (Amman and Hammerstedt, 1980) and acrosome status (Giemsa stain) in post-thawed semen were calculated. Number of motile spermatozoa or sperm with damage acrosome was counted in field of 200 spermatozoa then a percentage was calculated.

Conception rate (%):

Total of 100 sexually mature buffalo cows were divided into 10 groups, 10 animals in each group. Each buffalo cow in heat was artificially

inseminated with semen thawed at 55°C/15 sec and containing different types and levels of antioxidants.

Statistical analysis:

Data were statistically analyzed by the methods of analysis of variance according to Snedecor and Cochran (1982). Duncan's multiple range test was used to test the differences among means (Duncan, 1955).

The percentage values were subjected to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from transformed values to percentages.

RESULTS AND DISCUSION

Effect of types and levels of antioxidants on:

Sperm motility in post-thawed semen:

Results presented in Table (1) show that adding antioxidant to semen extenders significantly (P<0.05) increased percentage of sperm motility as compared to the control (un-supplemented), except for the highest level of AA (1.5 g/l), which did not differ significantly from that of the control. The best improvement in sperm motility was obtained with 1000 IU of catalase.

On the other hand, different effects were observed for types and levels of antioxidants on acrosome status (Table 1). The significant (P<0.05) reduction in percentage of spermatozoa with damage acrosome was observed with all catalase levels and the lowest level of GSH (0.4 mM) and AA (0.5 g/l). Also, the adding 1000 IU of catalase showed the lowest harmful effects on acrosome status.

In agreement with the present results of catalase addition, Mansour *et al.* (1982) found that extenders containing catalase give higher viability indices than the control of buffalo bulls. Extender containing 1000 IU catalase proved to be the superior one. The author observed the lowest fructose utilization in extender containing 1000 IU catalase, whereas the highest utilization was observed in extender containing low level of catalase (125 IU).

Table (1): Percentage of sperm motility and spermatozoa with damage acrosome in post-thawed semen supplemented with different types and levels of antioxidants.

Antioxidant supplementation		Sperm motility (%)	Damage acrosome (%)
Unsupplemented		40.0±1.36^{d}	11.9±3.65^{a}
Catalase	250 IU	46.4±2.01^{c}	9.4±3.56^{b}
	500 IU	53.6±3.39^{b}	7.7±2.91^{bc}
	1000 IU	62.1±3.59^{a}	6.0±2.27^{c}
Glutathione (GSH)	0.4 mM	60.7±3.99^{a}	7.4±2.81^{bc}
	0.8 mM	55.0±2.67^{b}	10.6±3.99^{ab}
	1.2 mM	52.1±3.59^{b}	12.7±4.80^{a}
Ascorbic acid (AA)	0.5 g/l	56.0±2.67^{b}	9.1±3.45^{b}
	1.0 g/l	47.9±1.84^{c}	11.3±4.27^{a}
	1.5 g/l	42.1±1.01^{d}	11.4±4.69^{a}

$^{a, b.....d}$: **Means denoted within the same column with different superscripts are significantly different at P<0.05.**

It has been found that the addition of catalase have positive effects in maintaining the motility and acrosome integrity of ram spermatozoa during liquid storage (Maxwell and Stojanov 1996) and catalase addition improved bull sperm survival in an egg yolk extender but not in milk extender (Foote *et al.*, 2002). This lake may be due to the high content in milk of the antioxidant casein (Taylor and Richardson, 1980). Also, Daader *et al.* (1982) found that the highest sperm viability with the highest level of catalse addition, being 38, 42, 50, 52 and 58% with levels of 0, 125, 250, 500 and 1000 U/ml, respectively. Moreover, El-Gaafary *et al.* (1990) and Fatouh and Abdou (1991) observed an increase in sperm motility of diluted bull semen supplemented with different levels of catalase to egg yolk extender in semen stored at 5°C.

It is worthy noting that all GSH levels had benefits on sperm motility, being the highest (60.7%) for level of 0.4 mM, which did not differ significantly from that of catalase at a level of 1000 IU, while the benefits of AA was at a level of 0.5 g/l only. In accordance with these results, Ahmed (2008) found that increasing level of antioxidant supplementation of AA more than 0.5 g/l or GSH more than 0.4 mM did not affect significantly sperm motility in post-thawed buffalo semen. In addition, Singh *et al.* (1995) noticed that pre-freeze fortification of Tris-based extenders with 2.5 mM of AA led to a significant augmentation in post-thaw motility of buffalo spermatozoa. Raina *et al.* (2002) reported that these vitamin C as natural antioxidant into semen diluents might prevent free radicals groups, which have induced oxidative damage to spermatozoa, and in turn inducing poor function and infertility (Delamirande and Gagnon, 1995).

Sperm motility percentage in semen thawed at different rates:

Results shown in Table (2) reveal that only antioxidant supplementation of catalase at a level of 1000 IU or AA at levels of 0.5 or 1.0 mM significantly ($P<0.05$) increased sperm motility percentage in post-thawed semen by different rates, being 38.3, 37.4 and 37.1%, respectively. However, the other types and levels of supplementation did not differ significantly from that of the control.

As affected by thawing rate, sperm motility percentage significantly ($P<0.05$) with a rate of 55^{o}C/15 sec than 35^{o}C/30 sec than 15^{o}C/60 sec (43.0, 32.11 and 27.0, respectively, Table 2). This means that increasing thawing temperature and decreasing thawing time resulted in the best results of sperm motility percentage in post-thawed semen (Table 2).

The insignificant effect of interaction between antioxidant group and thawing rate reflected in almost the higher sperm motility percentage post-thawing at a rate of 55^{o}C/15 sec than that with the other rates, being the highest (46-47%) with antioxidant supplementation of catalase at a level of 1000 IU or AA at levels of 0.5 and 1.0 mM (Fig. 1).

An appropriate rate of thawing in term of temperature and time is needed to avoid recrystallization in post-thawed semen. Recently, the best thawing rate with antioxidant supplementation to buffalo semen was suggested to be at 37°C for 30 sec (El-Nagar, 2007). The temperature higher than 37^{o}C was found to affect sperm motility, in particular for a longer time (Ziada *et al.*, 1992 and Rodriguez-Martinez, 2000).

Table (2): Sperm motility (%) in post-thawed semen supplemented with different types and levels of antioxidant and thawed with different rates.

Antioxidant group		Thawing rate (°C/sec)			Overall mean
Type	Level	15/60	35/30	55/15	
Unsupplemented		25.4±2.01	32.7±2.42	42.5±3.06	33.5±3.84[b]
Catalase	250 IU	25.0±1.54	32.9±2.14	40.0±2.18	32.6±4.1[b]
	500 IU	24.1±1.81	34.3±2.00	42.1±2.64	34.5±4.35[b]
	1000 IU	31.4±1.42	37.1±2.40	46.4±2.60	38.3±4.82[a]
Glutathione (GSH)	0.4 mM	27.9±2.40	33.6±3.21	43.6±3.20	35.0±4.41[ab]
	0.8 mM	29.3±2.29	43.3±3.16	44.3±3.32	35.9±4.53[ab]
	1.2 mM	26.4±2.10	30.0±2.88	37.9±2.85	31.4±3.96[b]
Ascorbic acid (AA)	0.5 g/l	28.6±2.60	36.4±2.60	47.1±2.58	37.4±4.71[a]
	1.0 g/l	27.9±2.40	36.4±2.60	47.1±2.85	37.1±4.68[a]
	1.5 g/l	24.3±1.69	31.4±2.61	39.3±2.29	31.7±3.99[b]
Overall mean		27.0±3.47[c]	32.11±4.29[b]	43.0±5.43[a]	

[a, b and c]: **Means denoted within the same column or row with different superscripts are significantly different at P<0.05).**

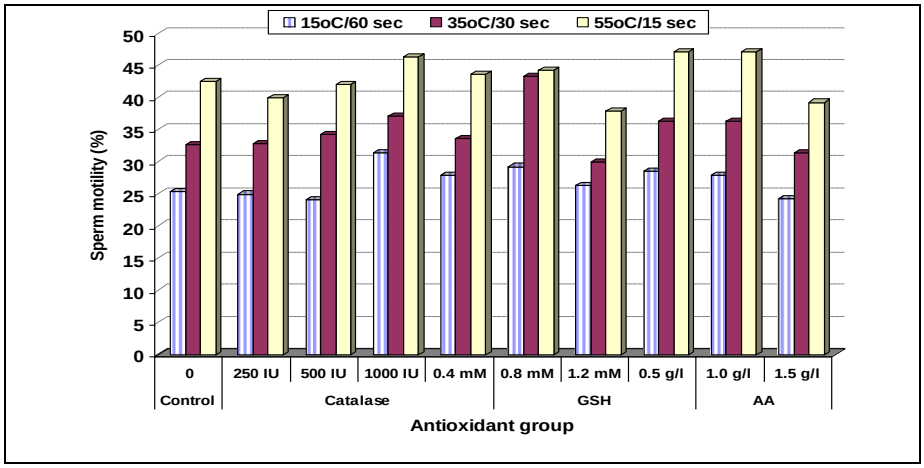

Fig. (1): Sperm motility percentage in frozen semen supplemented with different types and levels of antioxidant and thawed with different rates.

In this respect, Rodriguez-Martinez (2000) showed that the thawing time had an effect on the internal temperature of the straws thawed for 12 sec (4°C), while those thawed for 1 min reached an internal temperature of 36 °C. Therefore, the highest sperm motility percentage in this study was obtained by increasing thawing temperature up to 55°C for shorter time (15 sec). In bovine, Almquist *et al.* (1982) showed that sperm fertility was significantly higher when semen was thawed at 32-35°C for longer time (40-9 sec). Also, Mehrez (2001) indicated that thawing temperature at 37°C as an appropriate temperature for Friesian spermatozoa to resistant their motility for a long time.

Conception rate (CR):

The obtained results illustrated in Figure (2) indicated the highest CR (80%) of buffalo cows (8 cnceived/10 animals) inseminated by semen extended with Tris-based extender containing catalase at a level of 1000 IU as compared to 70% for those inseminated by semen extended with 500 IU of catalase, 0.4 or 0.8 mM of GSH, and 0.5 g/l of AA. On the other hand, the lowest level of catalase (250 IU) or the highest levels of GSH and AA resulted in 60% CR. While, the CR of the control cows inseminated with semen extended without antioxidant supplementation was the lowest, being 50%.

Similar trend was obtained by El-Nagar (2007), who found superiority of CR of buffalo cows inseminated by semen extended with camel skim milk (90%) or Tris-based (80%) extenders containing 100 IU of superoxide dismutase (SOD) as compared to 80 and 70% for the control animals without supplementation, respectively. Antioxidant supplementation maintained membrane integrity and intact acrosome as well as motility and livability of spermatozoa.

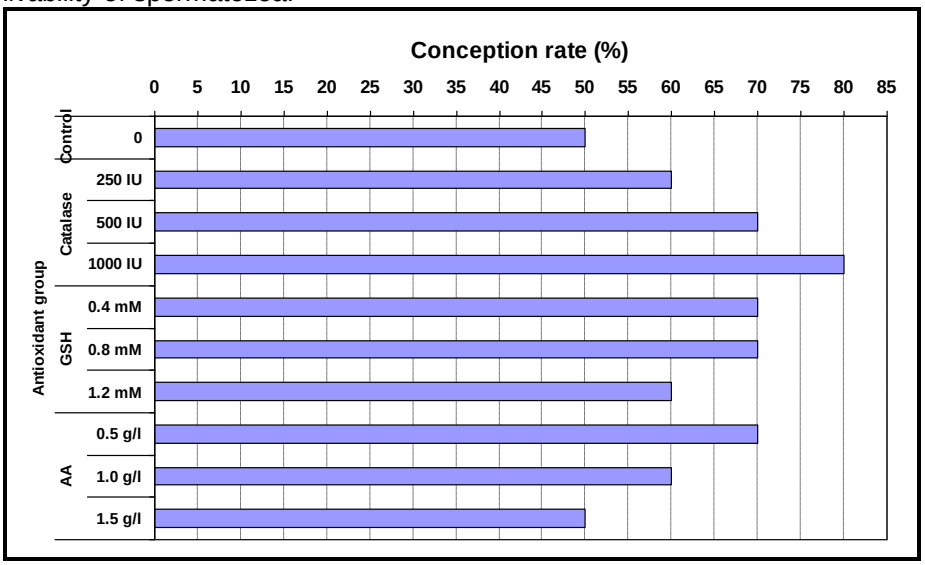

Fig. (2): Effect of frozen semen extended by Tris-egg-yolk supplemented with different types and levels of antioxidant on conception rate (%) of buffalo cows.

The highest CR of frozen semen extended with Tris-based extender with 1000 IU of catalase was mainly related to antioxidant supplementation, which was associated with the best improvement in percentages of sperm motility and spermatozoa with intact acrosome.

In accordance with increasing CR of buffalo cows inseminated with frozen semen extended with GSH, marked differences in CR of different types of extenders were indicated by several authors. Conception rate was 77% for buffalo cows inseminated by frozen semen diluted with skim milk lactose-citrate and egg yolk (Pavrithran *et al.*, 1972). In addition, Dhami and Kodagali (1990) reported that the fertility rate of frozen Surti buffalo semen

diluted in Tris-based extender was significantly (P<0.05) higher (42.69%) than that diluted in Citrate-based extender (39.78%).

Based on the obtained results of this study, adding Tris-based extender containing 1000 IU of catalase to frozen semen thawed at a rate of 55 ^{o}C for 15 sec showed the highest percentage of sperm motility and the best fertilizing capacity of buffalo spermatozoa.

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تأثير استخدام أنواع ومستويات مختلفة من مضادات الاكسدة على حيوية وحالة الاكروسوم للحيوانات المنويه للجاموس المصرى بعد التجميد والأسالة.

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تم تجميع السائل المنوى مرتين يوميا (100 قذفة) من 5 طلائق جاموسى ناضجة جنسيا (عمر 7-10 سنوات ,550-620 كجم وزن حى) خلال الفترة من يناير الى ابريل 2007 وذلك لدراسة تأثير أنواع ومستويات مختلفة من مضادات الأكسدة على حيوية الاسبرم وحالة الاكروسوم بعد اسالة السائل المنوى. وكان المخفف الاساسى المستخدم هو مخفف السترات وصفار البيض المحتوى على 7%جلسرول تم استخدام ثلاثة انواع من المضادات بثلاثة مستويات لكل منها كالتالى:-الكتاليز (500،250،1000 وحده دوليه) والجلوتاثيون (1.2،0.8،0.4 ميليمول) وحامض الاسكوربيك (1.5،1،0.5 جم/لتر) تمت مقارنة هذة المخففات بالمجموعة المقارنة التى ليس بها اى اضافات. تم تجميد السائل المنوى باستخدام النيتروجين السائل (-196°م) وتمت عملية الاسالة بالمعدلات التالية 60/15 ،30/35 ،15/55 °م /ثانية تم تقدير النسبة المئوية للحيوية والاكروسوم المحطم فى السائل المنوى بعد الاسالة. تم حساب معدل الاخصاب بتلقيح 100 جاموسة ناضجة

واظهرت النتائج مايلى:

1- أدى أضافة مضادات الاكسدة الى المخففات الى زيادة معنوية فى حيوية الحيوانات المنويه بالمقارنة بالمجموعة الضابطة ماعدا المستوى العالى من حامض الاسكوربيك(1.5جم/لتر) والذى لم يؤثر على حيوية الحيوانات المنويه وكان افضل تحسن فى مستوى حيوية الحيوانات المنويه (62.1%) مع استحدام 1000 وحده دوليه من الكتاليز

2- أدت جميع مستويات الكتاليز واقل مستوى من الجلوتاثيون (0.4 مليمول) أو حامض الاسكوربيك (0.5جم/لتر) الى تقليل نسبة الاكروسوم غير السليم بالمقارنة بالمجموعة الكنترول وقد حقق استخدام الكتاليز بمعدل 1000 وحده دوليه أعلى معدل فى تقليل نسبة الاكروسوم غير السليم (6%).

3- ارتفعت نسبة حيوية الحيوانات المنويه معنويا مع معدلات الاسالة على 55 °م لمدة 15 ثانية عن 35 °م لمدة 30 ثانية أو 15 °م لمدة 60 ثانية (43 ، 32.1 ،27% على التوالى)

4- أدى أضافة الكتاليز بمستوى 1000 وحده دوليه أو حمض الاسكوربيك بمستوى 0.5 أو1.5 جم /لتر الى زيادة معنوية فى حيوية الحيوانات المنويه فى السائل المنوى المسال بالمعدلات المختلفة (38.3 - 37.4 - 37.1 % على التوالى).

5- تم الحصول على أعلى معدل للاخصاب (80%) من الحيونات الملقحة بالسائل المنوى المخفف المحتوى على 1000 وحده دوليه من الكتاليز.

نستخلص من ذلك ان مخفف التريس الذى يحتوى على 1000 وحدة دولية من انزيم الكتاليز والمستخدم فى تخفيف السائل المنوى لطلائق الجاموس لكى يحفظ بالتجميد والمسال بمعدل 55°م لمدة 15 ثانية قد أعطى أعلى حيوية وأعلى معدل اخصاب وكذلك أفضل قدرة على التخزين بالتجميد .