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EFFECT OF SOME CRYOPROTECTANTS OR THEIR COMBINATIONS ON SPERM MOTILITY AND ENZYMATIC ACTIVITY IN POST-THAWED SEMEN OF FRIESIAN BULLS.

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ABSTRACT

The present study aimed to investigate the effect of various cryoprotective agents including 7% glycerol (GL), 7% dimethyl sulphoxide (DMSO), 5% ethylene glycol (EG), 5% acetamide (AC) or 5% lactamide (LA) and their combinations (3.5% GL+3.5 DMSO, 3.5% GL+2.5 EG, 3.5% GL+2.5% AC and 3.5% GL+2.5% LA) on post-thawing motility, recovery rate (freezability), acrosomal status and enzymatic activity of asprtate (AST) and alanin (ALT) transaminases, acid (ACP) and alkaline (ALP) phosphatases and lactic dehydrogenase (LDH) in post thawed semen. Semen was collected twice a week from five Friesian bulls (3-4 years old and 600-650 kg LBW) by means of an artificial vagina. Only semen with progressive motility ≥70% was pooled and used for different treatments. Semen was extended, frozen and thawed with lactose-yolk citrate extender containing different levels of cryoprotectants. Conception rate was also estimated by AI. Results revealed that semen extended with combination of 3.5% glycerol plus 3.5% DMSO showed significantly ($P<0.05$) the highest percentage of sperm motility (60.2%), recovery rate (75.6%) and the lowest percentage of damage acrosome (10%) as well as the lowest ($P<0.05$) activity of AST and ALT (16 and 7 $U/10^9$ sperm), ACP and ALK (105 and 50 $U/10^9$ sperm) and LDH (145 $U/10^9$ sperm) in post-thawed semen, respectively. On the other hand, semen extended with EG showed significantly ($P<0.05$) the poorest results as a single or in combinations of cryoprotectants.

In conclusion, using lactose-yolk-citrate extender containing a combination of 3.5% glycerol plus 3.5% DMSO during cryopreservation of bull semen showed the highest sperm motility, recovery rate and spermatozoa with intact acrosome in post-thawed semen, which reflected the highest conception rate of Friesian cows.

Keywords: Friesian, semen, motility, acrosome, cryoprotectants, freezing, thawing.

INTRODUCTION

The improvement of artificial insemination (AI) by frozen semen has important role in cattle breeding, especially in high intensive systems of production. Moreover, AI caused control reproduction and, in conjunction with accurate progeny testing, to improve the production of milk and meat. Other advantage of AI using frozen semen include more efficient genetic material are also obtained. Compared with natural mating, AI gives an increase in the number of offspring per sire (Hafez and Hafez, 2000).

The best preservation techniques to date of post-thaw survival are restricted to about 50% of the sperm population. It is important to recognize that the cryopreservation cycle includes the entire process from sperm preparation and dilution through the post-thaw maintenance of functional capability over an extended period, at each of these stages, spermatozoa can lose their ability to function normally. The optimum cryopreservation cycle must therefore to preserve, in largest possible proportion of live spermatozoa,

the acrosome integrity of different structures with different cryobiological requirements (Watson, 1995).

The final cryopreservation goal of semen is not only to maintain the initial motility but also to maintain the necessary metabolism to produce energy, plasma proteins to survive in the female reproductive tract at the time of fertilization, acrosomal enzymes for the penetration to the ovum, capacity of progressive movement and to prevent any damage which reduce life span of spermatozoa and its fertilizability. The major factor affecting the results of insemination with frozen-thawed semen including cooling, freezing, thawing, addition of cryoprotectants (Morris and Clark, 1987 and Aboagla and Trade, 2003), extension media, extension rate and spermatozoa damage due to the formation of internal ice crystals or to increased solute concentration in extension media or interaction of both of above physical factors (Lebouef *et al*. 2000). The level and type of cryoprotectants in semen diluent influence these events and their effects on the sperm cells during freezing (Watson, 2000 and Abdel-Khalek *et al*., 2008). Therefore, the cryoprotectants were added to extenders to maintain the sperm for damage during freezing process (Singer *et al*., 1995).

Many compounds have been tested for their efficacy as sperm cryoprotectants (Molinia *et al*., 1994), but most semen preservation protocols still favor glycerol (GL) in the cryoprotective media (Abdel-Khalek *et al*., 2008). In certain instances other cryoprotectants are possibly better, for example, dimethyl sulphoxide (DMSO) was preferred for buffalo bull (Abdel-Khalek *et al*., 2008) spermatozoa. Several studies demonstrated that ethylene glycol (EG) exhibited a cryoprotective effect (Rodrigues *et al*., 2004) and also acetamide (AC) and lactamide provided good protection to bull spermatozoa during freezing.

Spermatozoa subjected to cryopreservation are very sensitive to rapid reduction in temperature from 25°C to 5°C. This produces cold shock, a membrane transition phase behavior exhibited by biological membrane. Cold shock results in a loss of intracellular enzymes, a redistribution of ions a change in the membrane of acrosome and mitochondria, loss of motility and diminished metabolism (Watson, 1995 and Aboagla and Trade, 2003).

Therefore, the present study aimed to investigate the effect of various cryoprotective agents including 7% GL, 7% DMSO, 5% EG, 5% AC or 5% lactamide (LA) and their combinations (3.5% GL+3.5% DMSO, 3.5% GL+2.5% EG, 3.5% GL+2.5% AC and 3.5% GL+2.5% LA) on post-thawing motility, recovery rate (freezability), acrosomal status and enzymatic activity of asprtate (AST) and alanin (ALT) transaminases, acid (ACP) and alkaline (ALP) phosphatases and lactic dehydrogenase (LDH) in post thawed semen.

MATERIALS AND METHODS

The present study was conducted at the Department of Animal Production, Faculty of Agriculture, Mansoura University, Egypt. The experimental work was carried out in El-Gemmezah Animal Production Research Station, belonging to Animal Production Research Institute, during the period from April, 2007 till October, 2008.

Semen collection:

Semen was collected twice a week from five Friesian bulls (3-4 years old and 600-650 kg LBW) by means of an artificial vagina. Two successive ejaculates were obtained from each bull at each day of collection. All bulls were healthy and clinically free of external and internal parasites. Only semen with progressive motility ≥70% was pooled and used for different treatments.

Semen extension, freezing and thawing:

Immediately after collection, semen was extended with lactose-yolk citrate extender (LYCE) containing different levels of cryoprotectants as presented in Table (1). Extension of semen was done after collection in one step at a rate of 1 semen: 10 extender (v./v.) and then initial sperm progressive motility percentage was determined. Thereafter, extended semen with different types of cryoprotectants was kept at 5°C for 4 hours as an equilibration period, and then semen was packaged in straws (French straws, 0.5 ml) and frozen in liquid nitrogen at -196°C.

Table (1): Composition of lactose-yolk citrate extender (LYCE) containing different types of cryoprotectants and their combination used in semen dilution.

Ingredient	Type of extender								
	E1	E2	E3	E4	E5	E6	E7	E8	E9
Lactose-yolk citrate (ml)*	73	73	75	75	75	73	74	74	74
GL (ml)	7	-	-	-	-	3.5	3.5	3.5	3.5
DMSO (ml)	-	7	-	-	-	3.5	-	-	-
EG (ml)	-	-	5	-	-	-	2.5	-	-
AC (ml)	-	-	-	5	-	-	-	2.5	-
LA (ml)	-	-	-	-	5	-	-	-	2.5
Fresh egg yolk (ml)	20								
Penicillin (I.U/ml)	500								
Streptomycin (mg/ml)	500								

*** Lactose-yolk citrate composed of 2.9 g sodium citrate, 0.04 g citric acid, and 1.25 g lactose dissolved in 100 ml distilled water.**

Frozen semen was thawed by holding the straws at the closed end (not plugged end) and dipped in a water bath at 37°C for 30 seconds, then percentages of sperm progressive motility and spermatozoa with damage acrosome were determined immediately in post-thawed semen. Also, the enzymatic activities of asprtate (AST) and alanin (ALT), transaminases, acid (ACP) and alkaline (ALP) phosphatases and lactic dehydrogenase (LDH) were determined in thawed semen per 10^9 spermatozoa.

Semen evaluation:

Percentage of progressive sperm motility in extended semen post-dilution (initial) and in post-thawed semen was carried out and then, recovery rate (freezability) of the individual samples was calculated using the following formula:

Recovery rate (%) = (% Post-thawing motility/% initial motility) x100.

Assessment of the percentage of acrosomal damage was done according to Watson and Martin (1972) using buffered Giemsa solution. The percentage of acrosomal damage was calculated for 100 spermatozoa observed at random on each slide using immersion lens.

Enzymatic activities (U/10^9 spermatozoa):

Frozen-thawed semen samples were centrifuged at 1000 *g* for 15 minutes. The supernatant fluid (diluted seminal plasma) was collected then kept at -20°C till used for determination of AST, ALT, ACP and ALP enzymes. The activities of AST and ALT enzymes in the thawed-frozen semen were determined colourimetrically using QCA kits (Amposta, Spsin) according to **Reitman and Frankel (1957)**. The activities of ACP, ALP and LDH enzymes were determined colourimetrically by using commercial kits (Stanbio kit, Texas, USA).

Conception rate:

In the fertility trial, 40 normally cyclic cows were artificially inseminated with thawed-frozen semen extended with 3.5% GL+3.5% DMSO as compared to 7% glycerol, 20 cows in each group. Conception rate was estimated on the basis of pregnancy diagnosis by the rectal palpation after 60 days from date of insemination.

Statistical analysis:

The effects of treatment with different types of extenders on percentages of sperm motility and damage acrosome or on enzymatic activities were statistically carried out as one way design according to Snedecor and Cochran (1982) using SAS (1987). Duncan's Multiple Range Test (Duncan, 1955) was used to separate the means when the effect was significant at $P<0.05$. The percentage values of sperm progressive motility, livability, and abnormality were subjected to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from the transformed.

RESULTS AND DISCUSSION

Sperm characteristics in post-thawed semen:

Results presented in Table (2) revealed that the highest percentage of sperm motility, recovery rate and the lowest percentage of damage acrosome in post-thawed semen extended with single cryoprotectant were significantly ($P<0.05$) obtained with 7% GL or 7% DMSO. However, semen extended with combination of 3.5% glycerol plus 3.5% DMSO showed significantly ($P<0.05$) the highest percentage of sperm motility (60.2%), recovery rate (75.6%) and the lowest percentage of damage acrosome (10%) in post-thawed semen as compared to other combinations or single cryoprotectants. On the other hand, semen extended with EG showed significantly ($P<0.05$) the poorest results as a single or in combinations of cryoprotectants.

Table (2): Effect of extender type on sperm motility (%), recovery rate (%) and spermatozoa with damage acrosome (%) in post-thawed semen of Friesian bulls.

Extender type	Post-thaw Sperm motility (%)	Recovery rate (%)	Post-thaw damage acrosome (%)
7% GL	57.60±0.51^{b}	74.20±0.68^{a}	12.20±0.87^{c}
7% DMSO	57.20±0.67^{b}	75.20±1.37^{a}	12.00±0.96^{c}
5% EG	31.60±0.81^{e}	48.60±2.45^{f}	18.60±0.86^{a}
5% AC	42.80±0.59^{d}	57.40±0.98^{d}	15.60±0.88^{b}
5% LA	41.80±0.59^{d}	57.00±0.32^{d}	15.80±0.87^{b}
3.5 GL + 3.5% DMSO	60.20±0.49^{a}	75.60±0.40^{a}	10.00±0.32^{d}
3.5% GL + 2.5 EG	41.60±1.04^{d}	61.00±2.80^{c}	15.60±0.99^{b}
3.5% GL+ 2.5% AC	51.60±0.51^{c}	68.20±0.67^{b}	12.80±0.38^{c}
3.5% GL + 2.5% LA	51.20±0.59^{c}	67.80±0.77^{b}	12.80±0.59^{c}

a, b…….e: Means with different superscripts in the same column are significantly different at P≤0.05. GL: Glycerol. DMSO: Dimethyl sulphoxide. EG: Ethylene glycol. AC: Acetamide. LA: Lactamide.

In agreement with the best results obtained in this study, percentage of sperm motility in post-thawed buffalo semen and recovery rate were significantly (P<0.05) higher with GL or DMSO than with EG (Gabr, 2009). Also, Khalifa (2005) reported that extender containing 3% glycerol recorded the highest percentage of post-thawing motility while the lowest recorded with 3% ethylene glycol.

Zeidan *et al*. (2002) found that 2% glycerol or 4% DMSO maintained significantly (P<0.01) higher percentage of acrosomal integrity of frozen rabbit spermatozoa. Moreover, El-Gaaffary *et al*. (1993) found that 8% DMSO in diluent was more effective in protection of rabbit spermatozoa during freezing than did glycerol.

On the other hand, Singh *et al*. (1995) showed that both motility and percentage of live goat bucks spermatozoa were improved when in a combination of GL+DMSO. Moreover, El-Gaaffary *et al*. (1993) found that a medium contained 8% DMSO+2% GL was the best for protection of rabbit semen during freezing and post-thawing since it maintained the highest percentage of sperm motility, freezability and spermatozoa with normal acrosome. Abd-El-Salam (2002) and Zeidan *et al*. (2002) found that 2% glycerol and 4% DMSO significantly decreased the percentage of post-thawing acrosomal damage of frozen rabbit spermatozoa. Recently, Gabr (2009) found that higher percentages of post-thawing motility and recovery rate and the lowest sperm abnormality in buffalo semen diluted with a combination of glycerol plus DMSO.

Storage of bull semen at low temperatures caused structural damage as result of cold shock. The changes involved damage to the plasma membrane over the acrosome and the outer acrosomal membrane and damage to the plasma membrane of the middle piece. These changes are followed by decrease in the proportion of spermatozoa with intact acrosome. Therefore, the morphological characteristics of sperm acrosome with initial motility, gives the best indication, so far, of initial quality, especially for frozen semen. Penetrating cryoprotectants are used to preserve spermatozoa

viability during freezing. Their mode of action is mostly to reduce or prevent ice crystal formation. The osmotic effects of molar concentrations of cryoprotectants may result in membrane damage either during the introduction of cryoprotectants or on its removal depending on the permeability of the cell membrane to the cryoprotectant (Watson, 1995).

Enzymatic activities in post-thawed semen:

Activity of transaminases (AST and ALT):

Results presented in Table (3) revealed that the lowest AST and ALT activities in post-thawed semen extended with single cryoprotectant were significantly (P<0.05) obtained with 7% GL or 7% DMSO. However, semen extended with combination of 3.5% glycerol plus 3.5% DMSO showed significantly (P<0.05) the lowest activities of AST and ALT in post-thawed semen as compared to other combinations or single cryoprotectants. On the other hand, semen extended with EG showed significantly (P<0.05) the highest AST and ALT activities as a single or in combinations of cryoprotectants.

Activity of transaminases (AST and ALT) in semen is a good indicator of semen quality. Good quality semen was characterized by lower AST and ALT activity (Taha *et al.,* 2000). The present results are in agreement with those obtained by Zeidan *et al*. (2002) and Khalifa (2005), who found that a combination of 2% GL+2% DMSO recorded the lowest AST and ALT activities compared with 4% GL, 2% GL+2% AC, 2% DMSO+2% AC and 2% GL+2% DMSO+2% AC. In contrast, Gabr (2009) found that activity of AST in post-thawed semen was significantly (P<0.05) lower in post-thawed semen extended with EG than with glycerol or DMSO, being the highest with DMSO. However, activity of ALT in post-thawed semen was highest with DMSO, moderate with GL and the lowest with EG.

The obtained results regard to activity of AST in post-thawed bull semen was almost higher than ALT activity as estimated by several authors in seminal plasma of Friesian bulls (El-Sherbieny, 2004) and in buffalo semen (El-Nagaar, 2008).

Table (3): Means and standard errors of transaminases (AST and ALT) activity (U/10^9spermatozoa) in frozen bull semen as affected by different extender types.

Extender type	Transaminases activity (U/10^9spermatozoa)	
	AST	ALT
7% GL	18.40±0.52^{e}	10.00±0.72^{e}
7% DMSO	17.60±0.98^{e}	9.40±0.67^{e}
5% EG	38.80±3.86^{a}	16.00±0.71^{a}
5% AC	22.00±0.96^{c}	11.00±0.72^{d}
5% LA	23.80±1.08^{c}	13.00±0.70^{bc}
3.5 GL + 3.5% DMSO	16.00±0.91^{f}	7.00±0.72^{f}
3.5% GL + 2.5 EG	29.80±2.77^{b}	14.00±0.75^{b}
3.5% GL+ 2.5% AC	20.00±0.72^{d}	10.00±0.70e
3.5% GL + 2.5% LA	21.40±1.23^{cd}	12.00±0.71^{cd}

a, b…….f: Means with different superscripts in the same column are significantly different at P≤0.05. GL: Glycerol. DMSO: Dimethyl sulphoxide. EG: Ethylene glycol. AC: Acetamide. LA: Lactamide.

Activity of acid (ACP) and alkaline (ALP) phosphatases and lactic dehydrogenase (LDH):

Results presented in Table (4) revealed that the lowest ACP, ALP and LDH activities in post-thawed semen extended with single cryoprotectant were significantly (P<0.05) obtained with 7% GL or 7% DMSO. However, semen extended with combination of 3.5% glycerol plus 3.5% DMSO showed significantly (P<0.05) the lowest activities of ACP, ALP and LDH in post-thawed semen as compared to other combinations or single cryoprotectants. On the other hand, semen extended with EG showed significantly (P<0.05) the highest ACP, ALP and LDH activities as a single or in combinations of cryoprotectants.

These results are in agreement with those of Zeidan *et al*. (2002), who found that activity of ACP and ALP was significantly (P<0.05) lower in post-thawed semen extended with 4% DMSO and 2% glycerol+2% DMSO than with other cryoprotectants. On the other hand, Gabr (2009) revealed that activity of ACP in post-thawed buffalo semen was significantly (P<0.05) lower in post-thawed semen extended with GL than with DMSO or EG.

Based on the foregoing results, adding a combination of 3.5% glycerol plus 3.5% DMSO as cryoprotectants to lactose-yolk citrate extender during cryopreservation of bull semen showed the best motility, recovery rate and intact acrosome, and maintained enzymatic activity in post-thawed semen for use in artificial insemination (AI) programs of cattle breeding.

Table (4): Means and standard errors of activity (U/10^9spermatozoa) of acid and alkaline phosphatases as well as lactic dehydrodenase in frozen bull semen as affected by different extender types.

Extender type	Enzyme activity (U/10^9spermatozoa)		
	Acid phosphatase	alkaline phosphatase	lactic dehydrogenase
7% GL	149.0±1.87^{e}	59.0±1.14^{e}	278.2±2.08^{b}
7% DMSO	147.0±1.84^{e}	60.0±1.13^{e}	275.4±2.58^{b}
5% EG	334.0±1.85^{a}	170.0±1.15^{a}	313.6±4.36^{a}
5% AC	190.0±1.87^{c}	106.0±1.14^{c}	283.0±2.09^{b}
5% LA	192.0±1.84^{c}	109.6±1.38^{c}	285.9±2.55^{b}
3.5 GL + 3.5% DMSO	105.0±1.85^{f}	50.0±1.15^{e}	245.6±1.78^{d}
3.5% GL + 2.5 EG	225.0±1.87^{b}	116.0±1.13^{b}	291.0±5.18^{b}
3.5% GL+ 2.5% AC	170.0±1.84^{d}	90.0±1.15^{d}	253.4±7.90^{c}
3.5% GL + 2.5% LA	173.0±1.86^{d}	92.0±1.14^{d}	258.2±2.39^{c}

a, b…….f: Means with different superscripts in the same column are significantly different at P≤0.05. GL: Glycerol. DMSO: Dimethyl sulphoxide. EG: Ethylene glycol. AC: Acetamide. LA: Lactamide.

Conception rate:

The obtained results indicated slightly higher CR of cows inseminated with semen extended with a combination of 3.5% glycerol plus 3.5% DMSO (which showed the best results) than that contained 7% glycerol (75 *vs.* 70%), however, the difference was not significant. Such trend indicated that replacing the 50% of glycerol in the control extender (7% GL)

by DMSO improved CR of cows. This effect may be attributed to fewer changes, morphologically and functionally (Chatterjee and Gagnon, 2001) in bull spermatozoa during cryopreservation, when it extended with a combination of GL and DMSO as cryoprotectants.

Table (5): Conception rate (%) of cows artificially inseminated with semen extended with 7% glycerol compared with 3.5% glycerol plus 3.5% DMSO.

Extender type	Inseminated cows (n)	Conceived cows (n)	Conception rate (%)
7% GL	20	14	70
3.5% GL+3.5% DMSO	20	15	75

Storage of bull semen at low temperatures caused structural damage as result of cold shock. The changes involved damage to the plasma membrane over the acrosome and the outer acrosomal membrane and damage to the plasma membrane of the middle piece. These changes are followed by decrease in the proportion of spermatozoa with intact acrosome and increase in the release of enzymes into extracellular medium. Therefore, the morphological characteristics of sperm acrosomes and enzyme concentrations in the extracellular medium, with initial motility, give the best indication, so far, of initial quality, especially for frozen semen. However, the excessive amount of time necessary to carry out these tests prevents its routine use and most workers in the AI field prefer to use the viability of spermatozoa following incubation at 37ºC, and direct visual microscopic estimation. The prolongation of thawing-incubation at 37ºC for up to 2hours increased significantly ($P<0.05$) the percentage of acrosomal damage of bull spermatozoa with different types of cryoprotectants. These results are in agreement with those of Zeidan *et al*. (2002) and Gabr (2009). Penetrating cryoprotectants are used to preserve spermatozoa viability during freezing. Their mode of action is mostly to reduce or prevent ice crystal formation. The osmotic effects of molar concentrations of cryoprotectants may result in membrane damage either during the introduction of cryoprotectants or on its removal depending on the permeability of the cell membrane to the cryoprotectant (Watson, 1995).

In conclusion, using lactose-yolk-citrate extender containing a combination of 3.5% glycerol plus 3.5% DMSO during cryopreservation of bull semen showed the highest sperm motility, recovery rate and spermatozoa with intact acrosome in post-thawed semen, which reflected the highest conception rate of Friesian cows.

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تأثير بعض مواد الحماية من التجميد أو مخاليطها على حيوية الحيوانات المنوية والنشاط الانزيمي بعد التجميد والاسالة في طلائق الفريزيان

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تهدف هذه الدراسه لتقييم تأثير بعض مواد الحمايه من التجمبد على حيوية الحيوانات المنوية بعد التجميد والاسالة ومعدل الاسترداد وسلامة الأكروسوم والنشاط الانزيمي لكل من AST, ALT, ACP, ALP, LDH. تم جمع السائل المنوى مرتين اسبوعيا من خمس طلائق فريزيان عمر 3-4 سنوات بمتوسط وزن 600-650 كجم بواسطة المهبل الصناعى. تم تخفيف وتجميد عينات السائل المنوى ذات الحركة التقدمية ≥ 70% بمخفف اللاكتوز – صفار البيض – سترات مع اضافة مستويات مختلفة من مواد الحماية (7% جلسرول - 7% داي ميثيل سلفوكسيد - 5% ايثيلين جليكول - 5% اسيتاميد - 5% لاكتاميد –مخلوط من 3.5% جلسرول + 3.5% داى ميثيل سلفوكسيد – 3.5% جلسرول + 2.5% ايثيلين جليكول - 3.5% جلسرول + 2.5% اسيتاميد و 3.5% جلسرول + 2.5% لاكتاميد). كما تم حساب معدل الخصوبة باستخدام السائل المنوى المخفف ب 7%جلسرول ومخلوط 3.5% جلسرول+ 3.5% داى ميثيل سلفوكسيد. بينت النتائج وجود فروق معنوية على مستوى 5% بين مواد الحماية المختلفة حيث أظهر السائل المنوى المخفف بمخلوط من 3.5% جلسرول+3.5% داى ميثيل سلفوكسيد أعلى نسبة حيوية للحيوانات المنوية (60.2%) ومعدل الاسترداد (75.6%) وأقل نسبة من الحيوانات المنوية ذات الأكروسوم المشوه (10%) بعد الاسالة. أيضا أظهر السائل المنوى المخفف بمخلوط من 3.5% جلسرول+3.5% داى ميثيل سلفوكسيد أقل نشاط أنزيمى لكل من AST, ALT, ACP, ALP, LDH وكان معدل الخصوبه أعلى بصورة غير معنوية في الحيوانات الملقحة بالسائل المنوى المخفف بمخلوط 3.5% جلسرول + 3.5% داى ميثيل سلفوكسيد.

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