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AMILORATING EFFECTS OF CYSTEINE, GLUTATHIONE AND TAURINE DURING FREEZABILITY OF RAM SPERMATOZOA

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ABSTRACT

Ram sperm have a higher ratio of polyunsaturated/saturated fatty acids and a lower ratio of cholesterol/phospholipids molar than other species, which renders the sperm much more vulnerable to oxidative damage caused by reactive oxygen species (ROS) .The aim of the current study was to evaluate the effects of cysteine, glutathione and taurine on the freezability of ram spermatozoa and biochemical parameters. Semen samples from ten mature mixed Barki rams were used in this study. Pooled semen samples were divided randomly into 7 aliquots one was diluted with a Shotor extender as a control basal diluent while others in addition to basal extender L-Cysteine, L- reduced glutathione and taurine were added by two levels for each to present seven experimental groups. Diluted semen cooled to 5^{o}C then frozen by liquid nitrogen in 0.25 ml French straws. After 48 hr frozen, straws were then thawed individually at 37 ^{o}C for 30 sec. in a water bath then subjected to evaluation.

The obtained results showed that, addition of glutathione by a rate of 5mM was significantly increases motility than control group. Similarly, addition of taurine by a rate of 50mM significantly increased the viability index of cryopreserved semen samples.

Percentages of acrosomal defects were dramatically decreased ($P < 0.05$) with glutathione (10 mM) addition compared to control (9.00 ± 1.16 % and 21.00 ± 0.58 % respectively)

In regards to sperm membrane integrity, cysteine (5 mM) provided a greater protective effect (47.00 ±1.16) compared to control (%, 35.33 ± 0.88 %).

Antioxidant addition showed decreased enzyme leakage and significantly ($P < 0.05$) improved total antioxidant capacity (TAC) especially taurine (50Mm) compared to control group.

In regards to DNA integrity, taurine (50mM) provided a significant protective effect (98.51± 0.42 %) compared to control.

Findings of this study showed that addition of cysteine, glutathione and taurine to semen extenders, of greater benefit to quality of frozen-thawed ram sperm. However further investigation on their effect on fertilizing capacity and pregnancy rate still needed.

***Key word**: Shotor Diluent, Antioxidant, Cryopreservation, DNA integrity, Barki Ram Semen.*

INTRODUCTION

Artificial insemination has changed the small ruminant industry since it supports increased genetic improvement, better control of reproduction ,sexually transmitted diseases, dissemination of valuable genetics and preservation of the genetics of endangered breeds **(Cseh et al., 2012).** Development of techniques for the successful freezing of spermatozoa has progressively evolved over the past 60 years **(Kucuk et al., 2014).**The response of the spermatozoa to the cryopreservation varies among individual

males of the same species as well as in different species. In general, the spermatozoa of small ruminants are extremely sensitive to cryopreservation compared to other species **(Kucuk et al., 2014).**

Ram sperm have a higher polyunsaturated/saturated fatty acids ratio and a lower cholesterol/phospholipids molar ratio than other species, which renders the sperm much more vulnerable to oxidative damage caused by reactive oxygen species (ROS) **(Coyan et al., 2011) .**The controlled and spontaneous release of molecular oxygen, forming low concentrations of ROS, is required for the maintenance of the fertilizing ability and capacitation/acrosome reaction of spermatozoa, but excessive ROS impairs motility and capacity of fertilization **(Baumber et al., 2000)** .

Spermatozoa and seminal plasma possess an antioxidant system comprising taurine, reduced glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) to prevent oxidative damage and harmful effects of ROS. However, this antioxidant capacity of sperm, due to the small cytoplasmic component to scavenge oxidants, is limited. Thus, mammalian spermatozoa may, however, be insufficient in counteracting the damaging effects of ROS and lipid peroxidation (LPO) during the freeze thawing process **(Baumber et al., 2000) .** Addition of antioxidants could reduce the impact of oxidative stress induced by reactive oxygen species (ROS) generally during sperm storage **(Michael et al., 2009)**. A wide variety of antioxidants, such as glutathione (GSH), oxidized glutathione (GSSG), cysteine, taurine, hypotaurine, bovine serum albumine, trehalose or hyaluronan have been tested to minimize the damage caused by cooling and freezing-thawing on bull **(Uysal et al., 2007)**, stallion **(Ball et al., 2001)**, goat(**Salvador et al., 2006)** and ram **(Uysal et al., 2005)** semen.

The GSH is a ubiquitous molecule found at mM range in a number of cells, and is able to react with many ROS directly. The GSH is also a cofactor for GSH-Px that catalyzes the reduction of toxic H2O2 and hydroperoxides, protecting mammalian cells from oxidative stress, and the intracellular level of thiols in sperm is reduced by cryopreservation **(Bilodeau et al., 2009) .**

Cysteine has been shown to improve motility and morphology of post-thawed bull (**Bilodeau et al., 2009)** and (**Sarıözkan et al., 2009)** ram **(Uysal et al., 2007)** and goat (**Bucak and Uysal 2008**). Sperm, and to maintain the viability, the chromatin structure and membrane integrity of boar sperm during liquid storage at 5°C (**Szczesniak-Fabianczyk et al., 2003)**.

The sulfonated amino acid, taurine, present in both epididymal and oviduct fluid, is an important protector of cells against the accumulation of ROS when exposed to aerobic conditions **(Holmes et al., 1992)**.The beneficial effects of taurine in the semen preservation are due to that it is a very potent antioxidant **(Atessahin et al., 2008)**. **Fuahashi and sano (2005)** Demonstrated that, a marked improvement in the motility of ram and rabbit semen following the freeze–thawing process after addition of taurine to the freezing extenders. In recent years, taurine has been used as anti-oxidant in semen extenders and has been used in the cryopreservation of boar **(Lopes et al., 1998)**, bull **(Uysal et al., 2007)**, human (**Bucak et al., 2007)** , ram **(Atessahin et al ., 2008)**, and goat sperm **(Chen et al., 1993)** to improve motility, viability, membrane integrity, and fertility of spermatozoa by inhibiting lipid peroxidation

and protecting cells against accumulation of ROS **(Niasari-Naslaji et al., 2007)** .

The present study aimed to evaluate the protective effect of L- cysteine, glutathione reductase, taurine on ram semen during freezing .

MATERIALS AND METHODS

1. Animals and Semen Collection :

Semen samples from 5 mature mixed barki rams (1.5 : 2 years of age) were used in the study. The rams, belonging to the farm of Animal Reproduction Research Institute (ARRI), Agriculture Research Center, Giza, Egypt., were maintained under uniform feeding and housing conditions. Ejaculates were collected twice a week from the rams using an artificial vagina.

Only ejaculates of acceptable quality (motility,≥70% ,sperm concentration $\geq 3 \times 10^9$ sperm/ml were pooled and immediately used for dilution and cryopreservation) to minimize individual variation.

2- *Semen extending, freezing and thawing:*

The pooled ejaculates were diluted with the Shotor medium that composed of 214.6 mM tris, 64.2 mM citric acid, 66.6 mM glucose and 49.9 mM fructose, 20 ml egg yolk, 7% glycerol and glass-distilled water to 100 ml to having osmolality of 330 mOsm/kg and pH of 6.9, (freezing extender) according to (**Mohammed et al., 1998)** . Each mixed ejaculate was splited into seven equal aliquots and diluted at 37 ^{0}C by dilution rate, 200×10^6 sperm/ml) with the base extender (control group). More over others diluted with basal extender containing (5mM and 10mM) of cysteine hydrochloride (Loba Chemie PVT. Ltd.107, Wodehouse Road, Mumbai 400005, India) and (5mM and 10mM) of glutathione reductase(Sigma-Aldrich- Japan.), and (50mM& 100 mM) of taurine (Loba Chemie PVT. Ltd.107,Wodehouse Road, Mumbai 400005, India) respectively – for a total of seven experimental groups,. Diluted semen samples were aspirated into 0.25 ml French straws (IMV, L'Aigle, France), then suspended into liquid nitrogen vapour inside foam box 5 cm above the liquid nitrogen, for 15 min then plunged into liquid nitrogen for storage.

3- Semen assessment

After 48hrs frozen semen straws were thawed in a water bath at 37^{o}C for 30 second. Post-thawing sperm motility, viability and acrosomal integrity were assessed according to (**Mohammed et al., 1998) .**

4- Biochemical analysis

Extracellular aspartate-aminotransferase (AST),alanine-aminotransferase (ALT) and alkaline phosphatase (ALP) enzymes leakage during cryopreservation was assessed spectrophotometrically according **(Tietz ., 1976)** . Additionally, total antioxidant capacity (TAC) of the cryopreserved spermatozoa was measured using the method of (**Alvarez et al., 1987)** at 560 nm on a spectrophotometer and expressed as mM/L.

Statistical analysis:

All data were analyzed by using **Costat Computer Program** Cottort Software, and were compared by the least significant difference (LSD) at 5% levels of probability. The results were expressed as means ±SE.

RESULTS

Data presented in table (1) revealed that ,addition of 5mM glutathione to the freezing extender appeared to be the best additive concentration that improved (p< 0.05) the post thawing sperm motility (61.67 ±1.68%) as compared to the control one (46.67 ±1.68%).

While a concentration of **Taurine 50 mM** was the best one that improved (**P< 0.05**) sperm viability index as compared to the control (180.83±0.83, 145.83±2.21 respectively).

Data regarding the acrosomal defects, the best additive concentration that significantly decreased (p< 0.05) the acrosomal defects was glutathione 10mM (9.00 ± 1.16%) as compared to the control (21.00 ± 0.58%). The membrane integrity shown to be significantly improved (P< 0.05) than the control one (35.33±0.88%) with the best two additives concentration (cysteine 5mM& glutathione 10 mM) (47.00 ±1.16% &46.33 ± 1.21%) respectively.

Table (1). Effects Of Cysteine, Glutathione And Taurine On the freezability of ram spermatozoa

Parameters / Additives	Post Thawing Motility	Viability index	Acrosomal Defects	Membrane Integrity
Control	46.67 ±1.68 ^{c}	145.83 ± 2.21 ^{c}	21.00 ± 0.58 ^{a}	35.33 ± 0.88 ^{c}
Cystiene 5mM	56.67 ± 1.68 ^{ab}	166.67 ± 3.65^{b}	14.67 ± 1.21 ^{b}	47.00 ±1.16^{a}
Cystiene 10 mM	53.33 ± 1.68 ^{b}	167.50 ± 2.9^{b}	14.33 ± 1.21^{bc}	40.00 ±1.16 ^{b}
Glutathione reductase 5mM	61.67 ± 1.68 ^{a}	173.33 ± 3.65^{ab}	11.67 ±1.21^{bcd}	42.33 ±0.68^{b}
Glutathione reductase 10 mM	56.67 ± 1.68 ^{ab}	175.00 ± 4.91a^{b}	9.00 ± 1.16^{d}	46.33 ± 1.21^{a}
Taurine 50 mM	60.00 ± 1.68 ^{ab}	180.83 ± 0.83 ^{a}	11.33 ± 0.89 ^{bcd}	43.00 ± 1.16^{ab}
Taurine 100 mM	56.77 ± 1.68 ^{ab}	170.83 ±1.67 ^{b}	11.00 ± 1.16 ^{cd}	42.33 ± 1.21^{b}

*Values with different superscript letters in the same column are significantly different at (P <٠.05).

Mean values of some seminal plasma enzymes after cryopreservation as well as total antioxidant capacity are presented in Table (2).Addition of taurine (50 mM) was significantly (P< 0.05) decreased the enzyme leakage (AST,ALT,ALP) and significantly improved (TAC) cryopreserved ram semen samples (35.55 ± 1.20 U/L , 48.94 ± 0.36 U/L, 162.51 ± 0.34 U/L, 2.55 ± 0.12 mM/L) respectively as compared to the control one (44.41 ± 1.10 U/L , 72.26 ± 2.52 U/L, 184.43 ± 4.02 U/L , 1.21 ± 0.07 mM/L) respectively .

Table 2. Effects Of Cysteine, Glutathione And Taurine On the post thawing biochemical parameters of ram spermatozoa

Parameters **Additives**	**AST(U/L)**	**ALT(U/L)**	**ALP(U/L)**	**TAC(mM/L)**
Control	44.41 ± 1.10 [a]	72.26 ± 2.52 [a]	184.43 ± 4.02 [a]	1.21 ± 0.07 [a]
Cystiene 5mM	37.90 ± تراجع [bc]	50.94 ± 1.19 [cd]	171.69 ± 2.01 [b]	1.98 ± 0.10 [bcd]
Cystiene 10 mM	39.46 ± 0.23[b]	51.18 ± 1.09 [cd]	176.62 ± 0.39 [ab]	2.14 ± 0.11 [b]
Glutathione reductase 5mM	40.30 ± 0.12 [b]	56.82 ± 2.67 [bc]	162.64 ± 4.78 [c]	1.70 ± 0.10 [d]
Glutathione reductase 10 mM	36.22 ± 0.72 [c]	62.49 ± 4.22 [b]	179.62 ± 0.94 [ab]	1.77 ± 0.03 [cd]
Taurine 50 mM	35.55 ± 1.20 [c]	48.94 ± 0.36 [d]	162.51 ± 0.34 [c]	2.55 ± 0.12 [e]
Taurine 100 mM	36.21 ± 0.58 [c]	55.97 ± 2.36 [bcd]	179.16 ± 1.24 [ab]	2.06 ± 0.13 [bc]

*Values with different superscript letters in the same column are significantly different at (P < ٠.05).

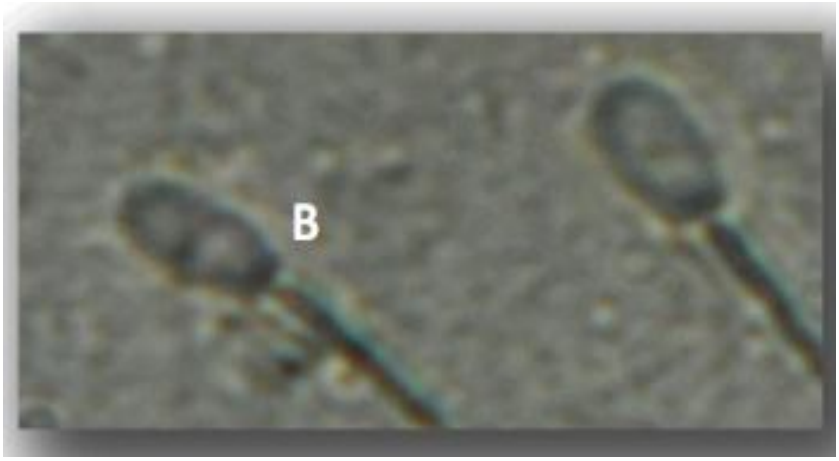

Figure 1: Post Thawing Ram Sperm Cryopreserved With Shotor Diluent Stained With Silver Nitrate Stain Showing A- Loosed Acrosome B-Detached Acrosome.

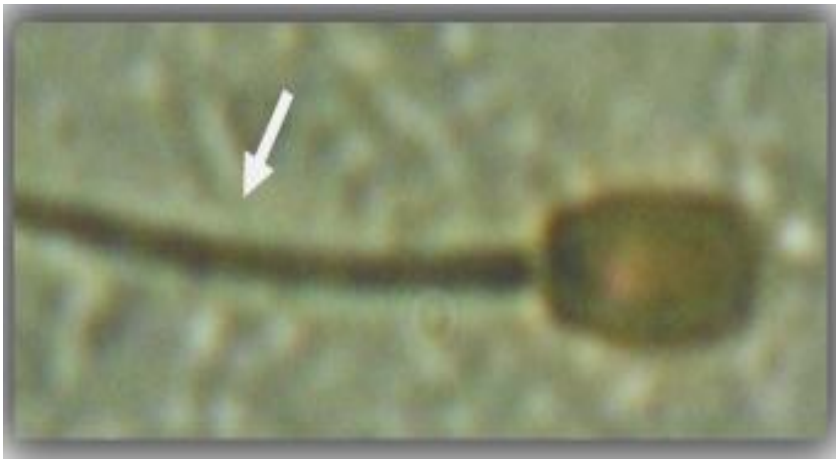

Figure 2: Post Thawing Ram Sperm Cryopreserved With Shotor Diluent And Stained With Silver Nitrate Stain Showing Intact Acrosome.

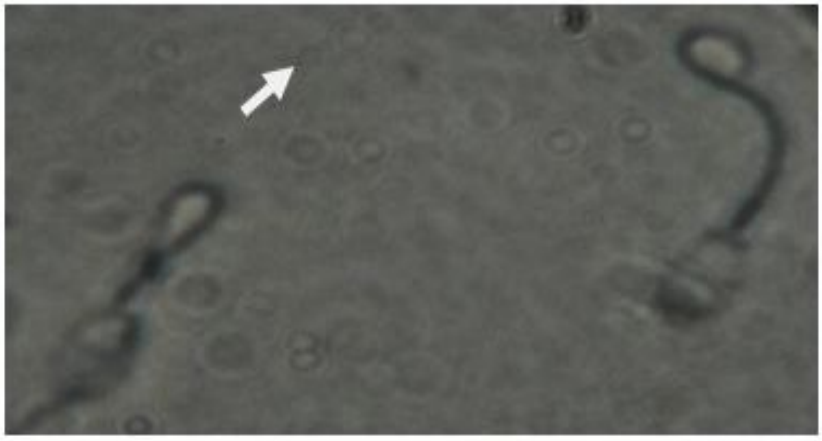

Figure 3: Post Thawing Ram Sperm Cryopreserved With Shotor Diluent Showing Swelling Of Tail After Exposure To Hypo-Osmotic Swelling Solution (HOST +VE).

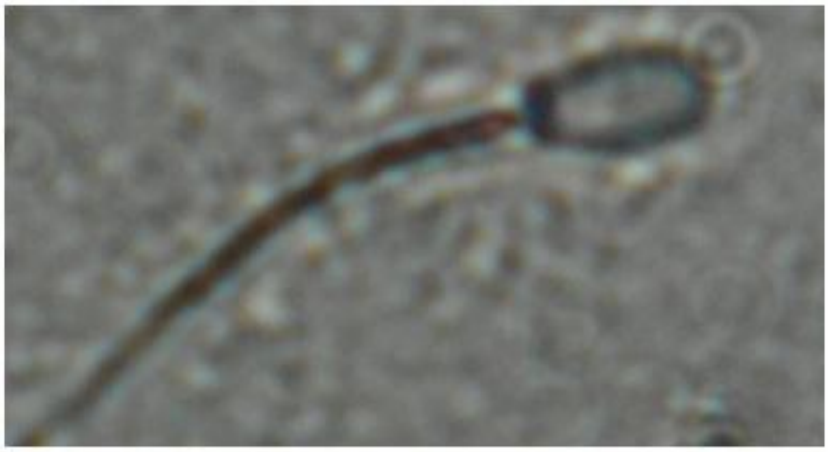

Figure 4: Post Thawing Ram Sperm Cryopreserved With Shotor Diluent Showing no Swelling Of Tail After Exposure To Hypo-Osmotic Swelling Solution (HOST -VE).

DISCUSSION

The sperm plasma membrane is rich in polyunsaturated fatty acids and is therefore susceptible to peroxidative damage with consequent loss of membrane integrity, decreased sperm motility, and eventually loss in fertility resulting from reactive oxygen species during aerobic incubation (**Bucak and Tekin ., 2007)** . Therefore, free radicals must be eliminated by supplementation with antioxidants such as GSH, cysteamine and taurine during the freezing-thawing or liquid storage of semen **[(Atessahin et al ., 2008 and Badr et al., 2014).**

In the present study, the results clearly indicated that supplementation of cysteine, glutathione and taurine to shotor extender prior to cryopreservation improved the post-thaw semen quality and biochemical finding of the cryopreserved barki ram semen , these result agrees with data of previous study carried out in bull (**Agarwal and Said ., 2003) .**

Regarding to the addition of cysteine to the semen extender, the present study demonstrated that cysteine exhibited positive impact on frozen sperm parameters and enhanced the biochemical characters. These results are in agreement with data obtained from several studies carried out in boar **(Lopes et al., 1998),** goat **(Zamfirescu and Şonea 2004)** ram **(Uysal et al., 2007)** bull (**Agarwal and Said ., 2003)** and buffalo **(Meister., 1994)** semen. The positive effect of cysteine over spermatic parameters is due to cysteine is an α-amino acid and is a precursor in the production of intracellular glutathione (GSH) which inactivates the reactive species of oxygen and catalyzes hydrogen or other superoxide detoxification **(Memon et al., 2011)** moreover it functions as a cofactor of glutathione peroxidase to destroy hydrogen peroxide (H_2O_2) (**Andreea et al., 2009) .** Additionally cysteine has cryoprotective effect on the functional integrity of axosome and mitochondria improving post thawed sperm motility and maintaining the morphological integrity over the head, middle and tail pieces in ram **(Uysal et al., 2007)** goat **[(Bucak and Uysal 2008) and Gadea et al., 2005)** bull **(Bilodeau et al., 2009)** and boar (**Szczesniak-Fabianczyk et al., 2003)**semen**.**

In agreement with **(Lopes et al., 1998) and** (**Agarwal and Said ., 2003)** finding**,** it has been shown that glutathione (GSH) can denote hydrogen atoms to repair damaged DNA therefor protect cells from DNA damage (**Gadea et al., 2005) .**

With respect to the addition of glutathione to the extender prior to the freezing **Agrawal and Said (2003)** showed that, cryopreservation in the glutathione-free extender wase associated with increased DNA deterioration. However, glutathione could be an important regulator of the scavenging system and one of the most important antioxidants in sperm cells **Gadea et al., 2005).** Therefore, the positive effect of GSH on the sperm freezability observed in the current study may be attributed to its ability to protect sperm against oxidative damage and reduction of oxidative stress-induced DNA oxidation and DNA fragmentation.

Taurine as antioxidant prevents efflux of cholesterol from the sperm membrane and Malonedialdehyde (MDA) production in diluents therefore it prevents premature

capacitating and acrosomal reaction (**Perumal and Rajkhowa 2013)**.

In regard to taurine addition to the extender, many studies **(Uysal et al., 2007 ; Fuahashi and sano 2005 and Lopes et al., 1998)** approved that, adding taurine to extenders before freezing causes improvement in forward motility of sperm after thawing in pig, bull, human, sheep and goat that was compatible with the present results .

AST and ALT are essential for metabolic processes which provide energy for survival, motility, and fertility of spermatozoa, and these transaminase activities in semen are good indicators of semen quality because they measure sperm membrane stability **(Corteel., (1980)**. High concentration of transaminase enzyme in the extracellular fluid due to sperm membrane damage and ease of leakage of enzymes from spermatozoa **(Gundogan., 2006) .**

In the present study, AST and ALT levels were lower in all antioxidant doses used in this study especially taurine (50mM) treated semen as it stabilizes the membrane integrity of acrosome, plasma and mitochondria membrane and flagella of the sperm, this finding come in agreement with **Gundogan (2006)** data .

In conclusion, findings emerging from this study clearly demonstrated that supplementation of semen extender with cysteine, glutathione and taurine exerted beneficial effects on quality of the frozen-thaw ram semen. These constructive effects appear due to improve the total antioxidant activities and diminish the acrosomal damage of the cryopreserved ram spermatozoa.

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الملخص العربي

تأثير الحمايه الناشئه من السيستين ، الجلوتاثيون والتوريين اثناء تجميد السائل المنوى للكباش

منى عبدالمقصود ، فهيم البهى ، منى شاكر و يوسف الصعيدى

نظرا لان الحيوان المنوى للكباش يحتوى على نسبه عاليه من الدهون الغير مشبعه ونسبه قليله من الكوليسترول عن باقى الكائنات الاخرى ولذالك فهو عرضه الى التلف بالاكسده.

ان الغرض من هذه الدراسه تقييم تدعيم مخفف الشوتور بكل من السيستين والجلوتاثيون والتوريين على درجة تجمد السائل المنوى للكباش.

تم تجميع السائل المنوى من كباش برقى بالغه ثم تم تقسيمها الى ٧ مجاميع وتخفيفها بمخفف الشوتور كمخفف اساسى وتم تدعيمه بكلا من السيستين والجلوتاثيون والتوريين بمستويين مختلفيين لكل مدعم. تم تجميد السائل المنوى بواسطة النتروجيين السائل بعد تخفيفه وتعبئته فى قصيبات ٤/١ مل. بعد ٤٨ ساعة تم اذابة القصيبات فى درجة حرارة ٣٧C٠ لمدة ٣٠ ثانية ثم تقييم السائل المنوى.

بينت النتائج ان تدعييم مخفف الشوتور بالجلوتاثيون بمعدل ٥ مل مول قد زاد معنويا من حركة الحيامن. وبالمثل تدعيمه بالتوريين بمعدل ٥٠ مل مول زاد معنويا من حيوية الحيامن. اما بالنسبه لسلامة القلنسوه فان الجلوتاثيون بمعدل ١٠ مل مول حافظ معنويا عليها وبالنسبه لغشاء الحيامن فان التدعيم بالسيستين بمعدل ٥ مل مول قد حافظ على سلامته. اما بالنسبة لانزيمات الاكسده فان فان التوريين بتركيز ٥٠ مل مول قد قلل من تسربها من الخلايا. تدعيم المخفف بالتوريين بتركيز ٥٠مل مول قد حافظ على الحامض النووى من التشوه.

من كل النتائج السابقه فان هذه الدراسه بينت ان تدعييم مخفف السائل المنوى بالسيستيين والجلوتاثيون والتوريين له تاثير نافع جدا على المحافظه على جودة السائل المنوى اثناء عملية التجميد والحفظ ولكن نحتاج الى دراسات اخرى لتقييم مدى خصوبة الحيامن .