EFFECT OF CYSTEINE SUPPLEMENTATION ON FREEZABILITY OF BALADIE GOAT SPERMATOZOA

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ABSTRACT

The aim of this study was to assay supplementation of cysteine into the traditional egg yolk extender for cryopreservation of buck spermatozoa. Semen ejaculates were collected from three fertile baladie bucks, aged 2.0 - 3 years using artificial vagina. Collected semen was divided into four aliquots; the first was diluted with Tris-egg yolk extender without any supplementation (control), while the others were diluted with Tris-egg yolk extender supplemented with cysteine at levels of 0.5, 2.5 and 5mM. Semen diluted at a rate of 1:4 and placed into a refrigerator at 5^{o}C for 4 h to equilibrate. At the end of equilibration period, extended semen was packaged in 0.25ml French straws and stored at –196^{o}C. Thereafter, frozen semen was thawed by dipping the straws into a water bath at 37^{o}C for 30 seconds. Percentages of progressive motility, live sperm, sperm abnormalities, plasma membrane and acrosome integrity were evaluated post dilution, equilibration period and post-thawing of goat semen.

The results revealed that there were a significant differences (P<0.05) of the percentages of sperm motility, live spermatozoa, sperm abnormalities and plasma membrane and acrosome integrity among post-dilution, post equilibration and post thawing of buck semen. Treatment supplemented with 2.5mM of cysteine led to significantly (P<0.05) improve the percentages of progressive motility, live spermatozoa, sperm abnormalities and plasma membrane and acrosome integrity of buck spermatozoa during different stages of cryopreservation compared to control and other levels of cysteine addition. In conclusion, supplementation of based Tris-egg yolk extender with 2.5mM of cysteine improves the percentages of progressive motility, live spermatozoa, sperm abnormalities and plasma membrane and acrosome integrity of frozen–thawed buck spermatozoa.

Key words: buck spermatozoa, cysteine, freezing, semen quality

INTRODUCTION

Egg yolk is a major constituent of extenders used for storage and cryopreservation of semen of domestic animals including bull, ram, goat and pig. The main advantage of egg yolk extender is the fraction of low density lipoprotein which protects the sperm during cryopreservation **(Amirat et al., 2005)**. However, wide variations in the

constituents of egg yolk make the beneficial effect difficult to assess **(Gil et al., 2003; Amirat et al., 2005)**. The artificial insemination (AI) in goats is biotechnological method providing augmentation of the genetic merit in goat flocks, successful preservation of superior male sperm will give the chance for future recalling even in the absence of those males **(Leboeuf et al., 2000)**.

The aim of storage of semen is to prolong the fertilizing capacity of spermatozoa by reducing or detaining their motility and metabolic reaction **(Evans and Maxwell, 1987)**. The main changes that occurred during storage included reduction in motility and morphological integrity of spermatozoa. It has been well defined that sperm freezing process has detrimental effect on post-thawed sperm quality **(Aitken et al., 1998; Salamon and Maxwell, 2000)**. Improvement of the extender is necessary because the main injury to sperm occurs during dilution and cooling **(Tasseron et al., 1977)**. Numerous studies have been carried out to evaluate fundamental biological properties of egg yolk in extender **(Watson, 1995; Martinez et al., 2006; EL-Sharawy, 2010; Noha Hussein, 2011)**. Several studies have been carried out to determine the effect of cysteine and glutathione on post-thawed sperm quality in different species **(Coyan et al., 2011; Sharafi et al., 2015; Uysal and Bucak, 2007; Atessahin et al., 2008; Zhandi and Ghadimi, 2014)**. Cysteine and glutathione have an important effect to diminish ROS during freeze–thawing process **(Uysal and Bucak 2007)**. Cysteine has cryoprotective effect on the functional integrity of acrosome and mitochondria improving post thawed sperm motility in many species, ram **(Uysal and Bucak, 2007)** goat semen **(Bucak and Uysal, 2008)**, bull semen **(Bilodeau, et al., 2001)**. **Andreea et al., (2010)** found that adding cysteine (10mM) and vitamin E (1mM) leading to the improve frozen buck semen. In opposite, **Perumal et al., (2011)** reported that addition of 5mM Cysteine and 5mM glutathione before freezing did not improve post-thaw progressive forward motility and total motility of the Jersey bull spermatozoa. The aim of this study was to evaluate the potential protective effect of cysteine on buck sperm characteristics including motility, livability, sperm abnormality, plasma membrane and acrosome integrity during different stages of cryopreservation.

MATERIALS AND METHODS

Experimental Animals

This study was carried out at the farm of Faculty of Agriculture, Kafr EL-Sheikh University. Three sexually mature bucks aged 2.0 – 3 years old with an average body weight of 60 kilogram were used during the period from March 2015 to July 2016. All bucks were healthy and free of internal and external parasites. Palpation of the external genitalia

showed that they were typically normal. The testicular tone was glandular, almost equal in size and moved freely up and down within the scrotal pouches. The animals were kept under natural photoperiod and balanced nutritional status. The rations offered to bucks adjusted to meet their maintenance requirements according to **NRC (2007)**. Fresh water was available at all the day.

Semen collection and evaluation

Following sexual preparation, semen was collected twice weekly by an artificial vagina from three bucks. Immediately after semen collection, ejaculates were held in a water bath at 37^{o}C until evaluated. Ejaculates having more than 75% mass motility were pooled in order to have sufficient semen for treatments and to eliminate the buck effect. On each collection day, good semen ejaculates were diluted with Tris-egg yolk extender, thereafter, the diluted semen was divided into four parts: the first part was used without any supplementation (control), while the others three parts were supplemented with 0.5, 2.5 and 5 mM of Cysteine. The sperm progressive motility was determined according to **Melrose and Laing (1970)**, live and abnormal spermatozoa were evaluated using eosin negrosin mixture prepared as described by **Hancock (1951)**, plasma membrane integrity of spermatozoa was according to **Jeyendran et al., (1984).** Acrosome integrity were assessed using Geimsa stain according to **Watson (1975)** in post-diluted, post-equilibrated and post-thawed semen.

Semen extender

Tris-based extender (cryopreservation control extender) consisted of 3.07g Tris (hydroxymethyl amino methane), 1.64g citric acid, 1.26g fructose, 15ml egg yolk, 5ml glycerol, 0.05 g streptomycin, 0.25 g linco-spectin and completed with bi-distilled water up to 100 ml. While, in other three extenders, cysteine was added to Tris-based control extender at concentrations of 0.5, 2.5 and 5mM. The dilution rate was 1:4.

Semen processing

Good ejaculates were further processed for freezing using 0.25ml French straws containing about 100x10^{6} motile sperm before freezing. The control Tris-egg yolk and Tris- cysteine extenders were kept warm in a water bath at 37^{o}C for 10 min. then they were placed into a refrigerator at 5^{o}C for 4 hours for gradual cooling and semen equilibration.

At the end of equilibration period, the extended semen was packaged in 0.25 ml French straws. Extended semen was kept in an ice water bath to keep its temperature at 5^{o}C. Straws were transferred into a processing canister and located horizontally in static nitrogen vapor 4 cm above the surface of liquid nitrogen for 10 minutes. The straws were then placed vertically in a metal canister and immersed

completely in liquid nitrogen container for storage at –196^{o}C. Freezing process was done as the method described by **Salisbury et al., (1978)**. For thawing, straws were dipped into a water bath at 37^{o}C for 30 seconds.

Statistical analysis

Data were statistically analyzed using a statistical software (SPSS, version 18.0). One-way analysis of variance was used to test the significance of extenders on the studied traits **(Steel et al., 1997)**. Means of the significantly affected traits were separated by Duncan Multiple Range Test **(Duncan, 1955)**.

Results

progressive motility

Results presented in Table (1) showed that semen supplemented with 2.5mM cysteine recorded the slight increase in the percentage of progressive motility of buck spermatozoa, compared to the control and 5mM extenders. While, semen supplemented with 0.5mM cysteine showed the lower value than the other treatments. During equilibrium period, addition of 2.5mM cysteine led to improve (P<0.05) sperm progressive motility compared to the control, while addition of 0.5 and 5mM cysteine resulted lower percentage of sperm motility compared to control. The percentage of frozen-thawed buck progressive motility were significantly (P<0.05) higher in extenders supplemented with 2.5 mM and 5mM cysteine compared to the control and 0.5mM cysteine extenders, being the best in semen supplemented with 2.5mM cysteine.

Table (1): Effect of supplementation of cysteine on the percentage of buck sperm progressive motility during different stages of cryopreservation

Stages	Control	Cysteine concentrations (Mm)		
		0.5	2.5	5
post – dilution	$70.5^{a} \pm 1.42$	$66.4^{b} \pm 1.18$	$72.3^{a} \pm 1.03$	$68.6^{ab} \pm 1.36$
post – equilibration	$60.5^{b} \pm 1.25$	$57.7^{b} \pm 1.40$	$65.0^{a} \pm 1.50$	$58.6^{b} \pm 1.52$
post – thawing	$29.9^{c} \pm 0.91$	$31.8^{c} \pm 1.01$	$41.4^{a} \pm 1.18$	$36.8^{b} \pm 1.01$

a, b and c: the different superscripts in the same row are significant at level 5%.

Live sperm

Post dilution, only the percentage of live spermatozoa was significantly (P<0.05) higher with 2.5mM cysteine extender compared to the control and the other cysteine extenders (Table 2). Post equilibrium period, the addition of different concentrations of cysteine led to increase the percentages of live spermatozoa compared to the control, being the best (P<0.05) in 2.5mM cysteine. Post freezing and thawing,

live sperm percentages in different cysteine extenders were significantly (P<0.05) higher compared to the control extender, being the best in 2.5mM cysteine extender.

Table (2): Effect of addition of cysteine on the percentage of buck live sperm during different stages of cryopreservation

Stages	Control	Cysteine concentrations (Mm)		
		0.5	2.5	5
post – dilution	$76.0^{b} \pm 1.40$	$71.0^{c} \pm 0.97$	$80.6^{a} \pm 0.79$	$75.1^{b} \pm 1.29$
post – equilibration	$60.2^{b} \pm 0.80$	$62.3^{b} \pm 1.38$	$75.5^{a} \pm 1.74$	$61.2^{b} \pm 1.40$
post – thawing	$36.0^{d} \pm 0.93$	$39.6^{c} \pm 1.25$	$51.5^{a} \pm 1.04$	$42.3^{b} \pm 0.47$

a, b, c and d: the different superscripts in the same row are significant at level 5%.

Sperm abnormality:

It is of interest to note that the addition of 2.5mM cysteine during different stages of cryopreservation (post dilution, post equilibrium period and post thawing) led to decrease the percentage of sperm abnormality compared to the control extender. While the addition of 0.5mM cysteine increased the percentage of sperm abnormalities compared to the other cysteine and control extenders (Table, 3).

Table (3): Effect of addition of cysteine on the percentage of buck sperm abnormality during different stages of cryopreservation

Stages	Control	Cysteine concentrations (Mm)		
		0.5	2.5	5
post – dilution	$8.4^{b} \pm 0.20$	$9.0^{a} \pm 0.13$	$8.1^{b} \pm 0.21$	$8.5^{ab} \pm 0.21$
post – equilibration	$8.4^{b} \pm 0.20$	$9.0^{a} \pm 0.13$	$8.1^{b} \pm 0.21$	$8.5^{ab} \pm 0.21$
post – thawing	$11.2^{ab} \pm 0.30$	$11.6^{a} \pm 0.15$	$10.0^{c} \pm 0.19$	$11.0^{b} \pm 0.13$

a, b and c: the different superscripts in the same row are significant at level 5%.

Plasma membrane integrity

Sperm plasma membrane integrity percentage was significantly (P<0.05) higher in extender containing 2.5mM cysteine when compared with control, 0.5mM and 5mM concentrations of cysteine at all stages of cryopreservation (Table,4). It was observed also, addition of 5mM cysteine significantly (P<0.05) improved the post thawed plasma membrane integrity percentage as compared to the control and 0.5 mM cysteine extenders.

Table (4) : Effect of addition of cysteine on the percentage of buck plasma membrane integrity during different stages of cryopreservation

Stages	control	Cysteine concentrations (Mm)		
		0.5	2.5	5
post – dilution	$73.2^{b} \pm 1.01$	$75.3^{b} \pm 1.85$	$80.5^{a} \pm 1.28$	$72.5^{b} \pm 1.53$
post – equilibration	$64.2^{c} \pm .951$	$68.6^{b} \pm 2.03$	$75.6^{a} \pm 1.44$	$62.1^{c} \pm 1.49$
post – thawing	$37.8^{c} \pm 1.30$	$37.1^{c} \pm 1.05$	$53.6^{a} \pm 1.18$	$43.1^{b} \pm 1.51$

a, b and c: the different superscripts in the same row are significant at level 5%.

Acrosome integrity

High ($P<0.05$) percentage of sperm acrosomal integrity was observed in 2.5mM cysteine extender at stages of equilibration and post freezing processes compared to the control and other levels of cysteine (Table, 4).

Table (5): Effect of addition of cysteine on the percentage of buck acrosome integrity during different stages of cryopreservation

Stages	**control**	**Cysteine concentrations (Mm)**		
		0.5	**2.5**	**5**
post – dilution	**$78.9^{ab} \pm 1.27$**	**$77.3^{b} \pm 1.76$**	**$81.8^{a} \pm 0.84$**	**$77.0^{b} \pm 1.25$**
post - equilibration	**$66.8^{b} \pm 1.63$**	**$70.2^{b} \pm 1.16$**	**$75.5^{a} \pm 1.52$**	**$67.0^{b} \pm 1.40$**
post - thawing	**$42.7^{b} \pm 0.91$**	**$41.9^{b} \pm 1.51$**	**$54.1^{a} \pm 0.58$**	**$44.9^{b} \pm 0.80$**

a, b : the different superscripts in the same row are significant at level 5%.

Discussion

Due to the high speed of the sperm, depending on the generation of energy by the mid-piece mitochondrial oxidative phosphorylation, a high concentration of free radicals are produced inside and outside the sperm cells **(Martin et al., 2004; Guthrie and Welch 2006)**. The increase of the production of reactive oxygen species (ROS) might damage the sperm cell membrane resulting in lower sperm motility and survival after storage at low temperatures (**Salamon and Maxwell, 1995)** which leads to diminishing the sperm penetration of the cervical mucus in vitro (**Gillan et al., 1997)**.

Addition of either enzymatic or nonenzymatic specific antioxidants would impact a beneficial reduction to the free radicals. Natural compounds containing thiol group are considered precursors of intracellular reduced glutathione biosynthesis. supplementation of cysteine to the semen extender prevents loss of sperm motility by inhibition of lipid peroxidation caused by ROS in frozen-thawed bull semen (**Bilodeau et al., 2000)**. Supplementing the freezing media of goat and ram semen by cysteine enhanced post-thaw motility (**Uysal**

and Bucak, 2007; Atessahin et al., 2008; Bucak and Uysal, 2008) and improved membrane integrity of boar sperm **(Funahashi and Sano, 2005)**.

Using high levels of cysteine did not protect goat sperm membrane and sperm viability during freezing as reported by **Kulaksiz and Daskin (2010)**. Moreover, **Uysal and Bucak (2007)** demonstrated that increasing doses of cysteine (15 mM) decreased post-thaw sperm abnormality and increased acrosomal damage in rams. In the present study, addition of cysteine exhibited positive effect on frozen buck sperm parameters such as motility, live sperm, sperm abnormalities and sperm membrane integrity and acrosome integrity. Furthermore, 2.5mM cysteine extender gives better results in frozen buck semen when comparing with other cysteine concentrations and control extenders. These findings are in agreement with **Funahashi and Sano (2005),** who reported that addition of low concentration of 5mM cysteine improved the viability and membrane integrity of boar spermatozoa during liquid storage.

Similarly, **Anghel and Zamfirescu (2010)** reported improvement in sperm cytological characteristics of frozen goat semen when tris extender supplemented with 5mM concentration of cysteine. Also, **Uysal and Bucak (2007)** reported that the best post thawing sperm parameters at 10 mM concentrations of cysteine in ram semen. In the meantime, **Kledmanee et al., (2013)** showed that the protective effect of cysteine on carp chilled sperm was attained by using levels up to 1 mM cysteine. Also, **Moustafa and Mona (2015)** observed that the best sperm survival in the tested cysteine level was found at the lowest concentration (1mM) in ram semen. Above this level, there found a significant ($P<0.01$) decline in sperm characteristics accompanied with increase free radicals and decreased antioxidant enzyme activity. This observation was also confirmed on post-thaw quality of Sahiwal bull semen (**Ansari et al., 2011)**.

Conclusion

In conclusion, the experimental present findings demonstrated that supplementation of semen diluent with 2.5mM cysteine improves semen characteristics (percentages of sperm motility, live sperm, sperm abnormalities, plasma membrane integrity, and acrosome integrity) when compare with control during cooling and freezing-thawing processes.

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الملخص العربى

تأثير إضافة السيستين على حفظ الحيوانات المنوية لذكور الماعزالبلدى بالتجميد

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*** قسم الإنتاج الحيوانى – كلية الزراعة – جامعة كفر الشيخ** قسم بحوث التكنولوجيا الحيوية – معهد بحوث الإنتاج الحيوانى – مركز البحوث الزراعية – مصر**

تم إجراء هذا البحث لدراسة تأثير إضافة السيستين بتركيزات مختلفة الى المخفف التقليدي (الترس –صفار البيض) لحفظ الحيوانات المنوية لذكور الماعز خلال مراحل الحفظ بالتجميد. تم جمع السائل المنوي بواسطة المهبل الصناعى من 3 تيوس أعمارها من 2-3 سنوات مرتين أسبوعيا خلال الفترة من مارس 2015 وحتى يوليو 2016م. تم خلط قذفات السائل المنوى التى تزيد حيويتها عن 75% وتخفيفها بمخفف الترس بنسبة 4:1 ثم يقسم إلى 4 أجزاء, الأولى تترك بدون اضافات (مجموعة ضابطة) بينما الثلاث أجزاء الأخرى أضيف اليها السيستين بتركيزات 0.5 ، 2.5، 5 مل مول. تم توضع عينات السائل المنوى المخفف فى الثلاجة على درجة حرارة 5 مئوية لمدة 4 ساعات (فترة موازنة) ثم تعبأ فى قصيبات

سعة 0.25 مل ثم توضع فى النيتروجين السائل على درجة حرارة -196 درجة مئوية ثم بعد التجميد يتم اسالتها فى حمام مائى درجة حرارته 37 درجة مئوية لمدة 30 ثانية. تم تقدير الحركة التقدمية ، نسبة الحى والشواذ وسلامة الغشاء البلازمى وغشاء الأكروسوم للحيوان المنوى وذلك بعد التخفيف، فترة الموازنة ، وبعد التجميد والإسالة.

أشارت النتائج إلى وجود فروق معنوية بإحتمال (0.05) في صفات السائل المنوي موضع الدراسة فى جميع مراحل الحفظ المختلفة (بعد التخفيف مباشرة، بعد فترة الموازنة والتجميد والإسالة). حقق المخفف المحتوي على 2.5مل مول من السيستين أعلى نسبة مئوية لكل من الحركة التقدمية والحيوانات المنوية الحية و سلامة الغشاء البلازمى والنسبة المئوية لسلامة الأكروسوم وأقل نسبة للحيوانات المنوية الشاذة مقارنة بالمجموعة الضابطة والتركيزات الأخرى من السيستين بينما أعطى المخفف المحتوى على 5 مل مول من السيستين أقل القيم فى جميع الصفات موضع الدراسة وكانت الاختلافات معنوية عند مستوى 5%. ويستخلص من هذه الدراسة أن إضافة 2.5 مل مول من السيستين إلى مخفف الترس مع صفار البيض يؤدى إلى تحسين صفات السائل المنوي لذكور الماعز بعد التجميد والإسالة وبالتالى التنبؤ بزيادة نسب الإخصاب.