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https://doi.org/10.5109/1854008

出版情報：九州大学大学院農学研究院紀要. 62 (2), pp.367-372, 2017-09-08. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **62** (2), 367–372 (2017)

Effect of Glutathione on Cryopreservation of Buck Spermatozoa

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(*Received April 28, 2017 and accepted May 10, 2017*)

The aim of this study was to assay supplementation of glutathione into the traditional egg yolk extender for cryopreservation of goat spermatozoa. Semen ejaculates were collected from three fertile baladie bucks, aged 2–3 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 glutathione at levels of 2, 4 and 6 mM. Semen diluted at a rate of 1:4 and placed into a refrigerator at 5°C for 4 h to equilibrate. At the end of equilibration period, extended semen was packaged in liquid 0.25 ml French straws and stored in liquid nitrogen at −196°C. Thereafter, frozen semen was thawed by dipping the straws into a water bath at 37°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 spermatozoa. The results revealed that there were significant differences ($P<0.05$) of various sperm characteristics (percentages of sperm motility, live spermatozoa and sperm abnormalities, plasma membrane and acrosome integrity) in post–diluted, post equilibration and post thawing of goat semen. Treatment supplemented with 6 mM of glutathione led to significantly ($P<0.05$) improve the percentages of progressive motility, live spermatozoa and sperm abnormalities, plasma membrane and acrosome integrity of buck spermatozoa during different stages of cryopreservation compared to control and other levels of glutathione addition. While the extender supplanted with 2 mM glutathione was recorded the lowest value of semen parameters approximately. In conclusion, supplementation of Tris–egg yolk extender used for buck semen extender during freezing–thawing process with 6 mM of glutathione improves percentages of progressive motility, live spermatozoa, sperm abnormalities, plasma membrane and acrosome integrity of frozen–thawed buck spermatozoa.

Key words: Buck Spermatozoa, Cryopreservation, Glutathione, Semen Extender

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 phospholipids 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). 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). As known, 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).

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 *et al.*, 2012 and El–Shamaa *et al.*, 2013). 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). Glutathione (GSH) has been shown to penetrate the cell membrane easily, enhancing the intracellular GSH biosynthesis both in vivo and in vitro and protecting the membrane lipids and proteins due to indirect radical scavenging properties (Topraggaleh *et al.*, 2014). It is also thought that GSH synthesis under in vitro conditions may be impaired because of deficiency of cysteine in the media, due to its high in stability and auto–oxidation to cysteine (Bucak and Uysal, 2008). Gadea *et al.* (2004) suggested that GSH is a tripeptide ubiquitously distributed in living cells and it plays an important role as an intracellular defense mechanism against oxidative stress. Also, they indicated that the process of freezing is associated with a significant reduction in GSH content in porcine semen. In addition, they indicated that glutathione a naturally occurring tri–peptide, works as an antioxidant in bovine semen to protect the sperm cells against reactive oxygen species (ROS) and it decreased in mammalian semen during cryopreservation process.

Therefore, the objective of this study to assess the goat semen characteristics in egg yolk extender supplemented by different levels of glutathione.

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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 2–3 years and their weight ranged between 55–60 kilogram were used in the current study during the period from (March 2016 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 during the experimental period according to NRC (2007). Fresh water was available during the experimental period.

Semen evaluation

Following sexual preparation, semen was collected twice weekly by artificial vagina from three bucks. Immediately after semen collection, ejaculates were held in a water bath at 37°C until evaluated. Ejaculates having good mass motility (more than 75%) were used and pooled. 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 without any supplementation (Control), while the others parts were supplemented with 2, 4 and 6 mM of Glutathione concentration.

Semen extender

Tris–egg yolk extender consisted of 3.07 g Tris (hydroxymethyl amino methane), 1.64 g citric acid, 1.26 g fructose, 15 ml egg yolk, 5 ml glycerol, 0.05 g streptomycin, 0.25 g linco–spectin and completed with bi–distilled water up to 100 ml (Control). While, the basal Tris–egg yolk extender was supplemented with 2, 4 and 6 mM of glutathione.

Semen processing

Good ejaculates were further processed for freezing using 0.25ml French straws containing about 100×10^6 motile sperm before freezing. The dilution rate was 1:4. The Tris–egg yolk and Tris– glutathione extenders were gently mixed and warmed up to 37°C in a bath water during semen extension. Vials containing the extended semen were placed in a water bath at 37°C and cooled gradually in a refrigerator at 5°C for 4 hours as an equilibration period.

At the end of equilibration period, the extended semen was loaded in 0.25 ml French straws. During packaging in straws, extended semen was kept in an ice water bath at 5°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°C. Freezing process was recorded as the method described by Salisbury *et al.* (1978). For thawing, straws were dipped into a bath water at 37°C for 30 seconds.

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 recorded 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.

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

The effect of various concentration of glutathione on progressive motility percentage of buck spermatozoa during different stages of cryopreservation are presented in Table (1). Glutathione concentrations at 6 mM had a significant ($P<0.05$) improving effect on motility percentage during different stage of cryopreservation when compared with the other treated groups. Moreover, addition of glutathione at 2 mM and 4 mM were recorded the better results for motility as compared with control group without significant effect (Table 1).

Live sperm

Live spermatozoa to EY–Tris extender at different concentrations on live sperm percentage at different stages of cryopreservation are presented in Table 2. Addition of 4 or 6 mM concentration of glutathione were

Table 1. Effect of glutathione on progressive motility (%) of buck semen during different stages of cryopreservation

Cryopreservation Stages	Control 0 mM	Glutathione concentrations		
		2 mM	4 mM	6 mM
Post–dilution	67.5 ± 1.11^{b}	68.0 ± 1.29^{b}	69.2 ± 1.53^{ab}	70.8 ± 1.53^{a}
Post – equilibration	51.6 ± 1.05^{b}	51.6 ± 2.47^{b}	59.2 ± 0.833^{a}	61.7 ± 2.47^{a}
Post – thawing	25.8 ± 1.53^{c}	26.0 ± 1.29^{c}	30.0 ± 2.58^{b}	36.7 ± 2.78^{a}

a, b and c: the different superscripts in the same row are significant ($P<0.05$).

Table 2. Effect of glutathione on live spermatozoa (%) of buck semen during different stages of cryopreservation

Cryopreservation Stages	Control 0 mM	Glutathione concentrations		
		2 mM	4 mM	6 mM
Post – dilution	70.1 ± 2.06[b]	70.3 ± .614[b]	76.0 ± 1.12[a]	76.3 ± 1.35[a]
Post – equilibration	58.0 ± 2.52[b]	58.8 ± 3.29[b]	66.3 ± 1.17[a]	66.6 ± 2.15[a]
Post – thawing	40.0 ± 4.44[b]	41.5 ± 2.69[b]	45.3 ± 3.76[a]	46.3 ± 1.96[a]

a, b and c: the different superscripts in the same row are significan ($P<0.05$).

Table 3. Effect of glutathione on sperm abnormality (%) of buck semen during different stages of cryopreservation

Cryopreservation Stages	Control 0 mM	Glutathione concentrations		
		2 mM	4 mM	6 mM
Post – dilution	8.0 ± 0.365	7.8 ± 0.307	7.7 ± 0.210	7.5 ± 0.401
Post – equilibration	8.2 ± 0.365	8.0 ± 0.365	8.2 ± 0.166	8.0 ± 0,365
Post – thawing	9.0 ± 0.365	8.5 ± 0.341	8.3 ± 0.210	8.3 ± 0.333

The differences between treatments are not significant ($P<0.05$).

Table 4. Effect of glutathione on plasma membrane (%) of buck semen during different stages of cryopreservation

Cryopreservation Stages	Control 0 mM	Glutathione concentrations		
		2 mM	4 mM	6 mM
Post – dilution	75.3 ± 1.42[cb]	77.5 ± 2.37[b]	76.6 ± 1.83[cb]	81.0 ± 1.50[a]
Post – equilibration	67.0 ± 1.48[c]	69.5 ± 2.32[b]	67.3 ± 2.95[c]	73.0 ± 1.67[a]
Post – thawing	53.2 ± 1.16[c]	56.1 ± 0.816[b]	56.0 ± 1.94[b]	59.3 ± 3.01[a]

a, b and c: the different superscripts in the same row are significant ($P<0.05$).

Table 5. Effect of glutathione on acrosome integrity (%) of buck semen during different stages of cryopreservation

Cryopreservation Stages	Control 0 mM	Glutathione concentrations		
		2 mM	4 mM	6 mM
Post – dilution	81.2 ± 0.980[b]	77.3 ± 0.954[c]	83.7 ± 1.17[b]	88.0 ± 1.00[a]
Post – equilibration	76.5 ± 2.34[bc]	73.5 ± 1.14[c]	78.7 ± 1.35[ab]	82.0 ± 1.36[a]
Post – thawing	66.8 ± 1.27[b]	62.2 ± 1.16[ab]	66.3 ± 2.09[ab]	67.8 ± 0.980[a]

a, b and c: the different superscripts in the same row are significant ($P<0.05$).

significantly ($P<0.05$) improved the percentage of live spermatozoa post – dilution and post – equilibration of goat semen extender. While, the lowest value of live spermatozoa percentage was recorded in the control group followed in order by semen extender containing 2 mM glutathione during the different stage of cryopreservation (Table 2).

Sperm abnormality

The percentage of sperm abnormality of buck semen supplemented with different concentrations of glutathione during different stages of cryopreservation is shown in Table (3). The experimental results in this study cleared that the sperm abnormality was not significantly ($P<0.05$) affected by any different levels of glutathione during post–dilution, post–equilibration and post–thawing. Sperm abnormality decreased progressively with increasing, adding glutathione concentrations and the control group recorded the highest level of sperm abnormality (Table 3).

Plasma membrane integrity

The effect of the different concentration of glutathione on plasma membrane integrity is set out in Table 4. Glutathione addition of 6 mM to buck goat semen had the highest number of plasmatic membrane during post–dilution, post–equilibration and post–thawing for extender goat semen followed in order by 2 mM, 4 mM then the lowest value was recorded in the control group (Table 4).

Acrosome integrity

The effect of glutathione addition at different concentrations on sperm acrosomal integrity percentage of buck spermatozoa at different stages of cryopreservation are given in Table (5). Higher percentage of sperm acrosome integrity was observed in extender containing 6 mM and 4 mM glutathione concentrations at all stages

of cryopreservation compared to the control group, the differences between groups were significant ($P<0.05$), while the lowest value were recorded in the extender containing 2 mM of GSH (Table 5).

DISCUSION

The experimental results revealed that progressive motility, live spermatozoa, plama membrane integrity and acrosome integrity percentages were improved by adding 6 mM GSH to extender. These results are in agreement with those reported by Uysal and Bucak (2007), who reported that glutathione at 5 mM showed more positive effect on motility of sperm followed in order by at 10 mM, 20 mM and control group. Whereas, a higher concentration of glutathione (10 or 20 mM) may have exhibited negative or toxic effect on progressive motility post–thawing spermatological indicators when compared to the 5 mM concentration of glutathione. Additionally, Bilodeau *et al.* (2001) found that the thiols such as glutathione prevented the loss of sperm motility in frozen–thawed bull semen. On the other hand, Noei Rozliqi *et al.* (2015) found that the extender semen containing 1.0 mM glutathione led to significantly ($P<0.05$) higher percentage of total progressive motility (50.75%) of spermatozoa compared to 0.5, 1.5, 2 mM glutathione extender. Therefore, a part of the beneficial effect of total motility at 1.0 mM glutathione may be related to improve plasma membrane integrity and functionality. Moreover, Topraggaleh *et al.* (2014) found that the decrease in superoxide anions, which found at addition of high level of glutathione (15 mM), was the main reason of increasing the motility of frozen–thawed buffalo spermatozoa. So, the potential cause of the decline in motility and fertility during hypothermic storage of liquid semen is an oxidative damage of spermatozoa (Ball *et al.*, 2001). Additionally, Ansari *et al.* (2014) found that the percentage of post thaw sperm motility was significantly ($P<0.05$) higher in extender containing GSH 0.5 mM (55.0 ± 0.0%) compared to extender containing glutathione 1.0 mM (46.7 ± 3.0%) and control group (43.3 ± 2.9%) in Sahiwal bull spermatozoa. Therefore, to maintain sperm for longer period cool and cryopreserved, it is necessary to dilute semen in a protective solution (Ax *et al.*, 2000). Evidence has shown that the rapid decline in intracellular GSH concentrations that occurs during the incubation of spermatozoa under aerobic conditions is not associated with an increase in GSSG concentrations (Bilodeau *et al.*, 2000).

Our results in this study indicated that addition of 4mM and 6mM to buck semen extender showed high significant ($P<0.05$) percentages of live buck spermatozoa compared by control and 2mM GSH, respectively. On the contrary, Oliveira *et al.* (2013) found that the viability spermatozoa for frozen equine semen was recorded the highest value in the extender containing 2.5 mM glutathione followed in order by control group, 5 mM, 7.5 mM and 10 mM, respectively. Also, Noei Razliqi *et al.* (2015) reported that the live sperm percentage in the different extender of buck semen containing 0.0, 0.5, 1.0, 1.5 and 2.0 mM GSH were 35.83, 36.58, 53.23, 50.98 and 36.35%, respectively. In the same trend, Ansari *et al.* (2014) observed that the viable spermatozoa percentage post – thawed was higher significantly ($P<0.05$) in Sahwil bull spermatozoa extender containing 0.5 mM in comparison with extender containing 1.0 mM GSH and control group. Furthermore, Luberda (2005) suggested that the protection by glutathione against oxidative damage is provided by its sulphydryl group, which can be presented in reduced (GSH) and oxidized glutathione (GSSH) forms. Also, Munsi *et al.* (2007) found that glutathione may stabilize the plasma membrane of spermatozoa during cryopreservation. On the other hand, Camara *et al.* (2011) reported that an addition of glutathione at high concentration (3.5 and 10 mM) had no beneficial effect on semen quality parameters, whereas the high concentration of glutathione may cause degradation of mitochondrial DNA. In the same trend, Ansari *et al.* (2010) found that addition of glutathione at 3 mM failed to improve the sperm viability, (Table 2).

The percentage of abnormal buck spermatozoa in this study was not significant effect by GSH concentration, but the lowest abnormal sperm percentage was recorded with 6 mM. These results are in agreement with those reported by Uysal and Bucak (2007), who found that addition of glutathione at 5 mM had the lowest percentage of sperm abnormality in ram frozen semen followed in order by10 mM, 20 mM and control group, respectively. Whereas, the cold shock of sperm cells during the freezing – thawing process is associated with oxidative stress induced by free radicals and thus free radicals are eliminated by antioxidant systems (Salvador *et al.*, 2006; Sanacka and Kurpisz, 2004), (Table 3).

Also, our results in this study are in agreement with those reported by Uysal and Bucak (2007), who found that the supplementation of glutathione at 5.0 mM was significantly recorded the better value of plasmatic membrane integrity followed in order by 20 mM, 10 mM and the lowest value was recorded in the control group in frozen ram semen. In the contrary, the current results study were higher than reported by Oliveira *et al.* (2013), Who reported that plasmatic membrane integrity in stallions semen was higher in extender containing 2.5 mM followed in order by control group, 5.0 mM, 7.5 mM, and 10 mM, respectively. In the same trend, Gadea *et al.* (2004) demonstrated that addition of 5 mM glutathione concentration to the freezing extender swine semen did not improve plasmatic membrane integrity. While, Gadea *et al.* (2005) found that an addition of 1 mM and 5 mM glutathione increased the percentage of plasma membrane integrity. Moreover, Ansari *et al.* (2014) reported that a higher ($P<0.05$) percentage of sperm with intact plasma membrane was observed in extender containing 0.5 mM compared to extender containing glutathione 1.0 mM and control group. Additionally, Bilodeau *et al.* (2000) observed that glutathione is present in semen and provides intracellular defense to sperm against the oxidative stress, whereas the freeze–thawing results in a significant reduction in the glutathione content of the semen and to provide a

protective role in maintaining the quality of sperm. Also, De Mercada *et al*. (2009) reported that glutathione prevents lipid peroxidation by enhancing sperm antioxidant capacity as well as its cryoprotectant role for stabilizing sperm membrane. So, these difference between results may cause by different extender, different animals and may be due to the different storage procedures. Furthermore, plasma membrane damage may be caused by several factors such as the availability of nutrition for decreasing spermatozoa, unsuitable environments live low temperature, the formation of lactic acid from metabolism residue that can decrease the pH, inappropriate diluting and chilling process, the formation of lipid peroxidation reaction, low quality semen as a lot of abnormal spermatozoa and the occurrence of cell damages were found (Beconi *et al*., 1993).

In the same trend, Mishra *et al*. (2010) reported that a glutathione plays an important role in scavenging reactive oxygen intermediates and other free radicals such as hydrogen peroxide with the help of glutathione reductase and it may protect the sperm from membrane damage by inhibiting lipid peroxidation.

The results in this study are in agreement with Uysal and Bucak (2007), who found that the glutathione at 5 mM had a significant ($P<0.001$) effect in maintaining post – thaw sperm acrosome integrity followed in order 20 mM, 10 mM and control group. Furthermore, Ansari *et al*. (2014) found that post–thawed percentage of acrosome integrity was significantly ($P<0.05$) higher in extender containing 0.5 mM when compared with extender containing GSH 1.0 mM (63.3 ± 2.1%) and control group (59.3 ± 3.1%). Moreover, Ansari *et al*. (2012) suggested that supplementation of the GSH in semen extender might improve the acrosome integrity due to protection against ROS molecules which produced from mitochondria, plasma membrane lipid peroxidation and dead / abnormal spermatozoa during freeze – thawing process. In the contrary, Mishra *et al*. (2010) found that the mean percentage of acrosome abnormality (1.1 ± 0.3%) was significantly ($P<0.01$) lower in the semen with 8 mM glutathione than with 2 mM (2.2 ± 0.2%) or 4 mM glutathione (1.6 ± 0.2%) or control group (2.4 ± 0.2%) after stored semen at 5°C. So, Sinha *et al*. (1996) suggested that GSH in a co–enzyme of 1, 3–diphosphoglyceric–aldehyde dehydrogenase leads to oxidation of triose phosphate to phosphoglyceric acid which is later reduced to pyruvic acid and then to lactic acid. The free radicals must be destroyed by the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase that damages the cell membrane and acrosome by lipid peroxidation during preservation (Cotran *et al*., 1989).

CONCLUSION

In conclusion, the experimental present findings demonstrated that supplementation of semen diluent with 6 mM glutathione improves semen characteristics (percentages of sperm motility, live sperm, sperm abnormalities, plasma membrane integrity, and acrosome integrity) when compared with the control group during freezing–thawing processes.

AUTHOR CONTRIBUTIONS

1) Study conception and design: B. EL–BEHNSAWY; E. EL–SIEFY; R. EL–HALAWANY and A. METWALLY
2) Acquisition of data: B. EL–BEHNSAWY; E. EL–SIEFY; R. EL–HALAWANY and A. METWALLY
3) Analysis and interpretation of data: E. EL–SIEFY; M. EL–SHARAWY; K. KUBOTA; N. YAMAUCHI and A. METWALLY
4) Drafting of manuscript: B. EL–BEHNSAWY; E. EL–SIEFY; M. EL–SHARAWY; K. KUBOTA; N. YAMAUCHI and A. METWALLY
5) Critical revision: M. EL–SHARAWY; K. KUBOTA; N. YAMAUCHI and A. METWALLY

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