EFFECT OF GLUTATHIONE SUPPLEMENTATION TO SEMEN EXTENDER ON COCKEREL SPERM CHARACTERISTICS IN SEMEN STORED AT 5°C FOR DIFFERENT PERIODS

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SUMMARY

The present study was conducted to evaluate the effect of glutathione (GSH) supplementation at levels of 0, 0.2, 0.4 and 0.6 mM to diluted semen stored for 0, 24, 48 and 72 h at 5°C on sperm characteristics and fertility rate (FR) of Inshas chickens. Effect of interaction between GSH level and storage period was not significant. Results revealed that progressive sperm motility (PSM), sperm livability (SL), sperm with curled tail (CT), sperm abnormality (SA) and acrosomal damage (AD) traits were improved by adding GSH to semen extender at all levels compared with no extender (control group). Semen diluted with 0.2 mM GSH showed the best results and semen without GSH supplementation (control) showed the poorest quality traits. Storage period had deleterious effect on all semen characteristics of cockerel spermatozoa. Percentages of PSM, SL and CT decreased, while those of SA and AD were increased by increasing storage period. The FR was higher for semen supplemented with 0.2 mM GSH (74.5%) than those with 0.6 mM GSH or free semen (59.9 and 59.2%, respectively). However, FR of semen extended supplemented with 0.4 mM GSH did not differ significantly when compared with other GSH levels, nor the control ones. The FR showed gradual reduction by increasing storage time, being the highest at 0 h and the lowest in semen stored for 72 h. The FR was almost higher for semen supplemented with 0.2 mM GSH and stored at each period compared with other semen treatments.

In conclusion, supplementation of GSH at a level of 0.2 mM to extender of cockerel semen stored at 5°C for up to 72 h is an appropriate level to improve semen characteristics and fertility rates of Inshas chicken spermatozoa.

Keywords: Chicken, semen, antioxidant, preservation, sperm characteristics

INTRODUCTION

Lipids as major compound in semen, particularly in sperm membrane, contribute also in sperm metabolism and their capacitatation and fertilizing ability of female gamete. This relationship was indicated in birds (Ansah and Buckland, 1982). In mammalian spermatozoa, adversely effects of lipid peroxidation (LPO) was reported in terms of losing fluidity of membrane, reducing function of acrosome, chromatin damage, and decreasing oocyte fusion by sperm (Long and Kramer, 2003). In mammals, membrane of spermatozoa has a high content of unsaturated fatty acids and sperm cells lack a significant cytoplasmic component containing antioxidants. Therefore, they are susceptible to LPO by O_2 and H_2O_2 (Storey, 1997). Increasing the LPO in sperm cell membrane, as a result of exposing to reactive oxygen species (ROS), causes breakdown of membrane, increasing abnormality as well as decreasing motility of spermatozoa. Finally, decreasing capacity of sperm cells to penetrate oocyte (Wang *et al.*, 1997 and Potts *et al.,* 1999).

Membrane of avian spermatozoa is rich in polyunsaturated fatty acid (PUFAs) and can easily undergo LPO in the presence of ROS (Thananurak *et al*., 2015). During *in vitro* storage, avian semen tended to produce high LPO concentrations, resulting in a partial or complete losing the fertilizing capacity (Wishart, 1984). After *in vitro* storage, a direct relationship was observed between compromised function of poultry sperm and LPO (Cecil and Bakst, 1993). Deleterious effects of LPO on sperm cells in poultry lead to morphological defects, low motility, and decreasing fertilizing ability (Long and Kramer, 2003). In sheep, a defense mechanism against LPO, which plays a important role in maintaining sperm function included many types of antioxidants (catalase, glutathione, GSH; glutathione peroxidase, GPx; superoxid dismutase, SOD) as reported by Bucak *et al.* (2008).

In chicken breeders, sperm enrichment in n-3 or n-6 PUFAs for improving sperm production and fertilizing ability by different oil sources have been reported by Cerolini *et al.* (2006). In bovine, the oil supplementation may increase LPO, which may affect sperm function. Also, different processing procedures or semen preservation (at cool or room temperature), and exposing spermatozoa used in artificial insemination to oxygen and visible light radiation could lead to formation of ROS, and negatively affect motility and genomic integrity of

spermatozoa (Bilodeau *et al*., 2001). To maintain sperm livability and motility, adding different types of antioxidants is vital under previous conditions (Bilodeau *et al*., 2001; Foote *et al*., 2002 and El-Sherbieny *et al*., 2006).

The aim of the current study was to determine the effects of addition of reduced glutathione at levels of 0, 0.2, 0.4 and 0.6 mM to the extender of cockerel semen stored for 0, 24, 48 and 72 h at 5°C, on sperm characteristics and fertility of Inshas strain.

MATERIALS AND METHODS

The current study was carried out at Sakha Experimental Research Station, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture.

Experimental chickens:

Ten mature cockerels of local breed (Inshas strain), 9-10 months of age and 2.30 -2.53 kg live body weight were used in this experiment.. All cocks were kept in an individual cages (60x50x75 cm), provided with laying mash diet containing 15.5% crude protein and 2700 kcl ME per kg diet. Water was available all daytime.

Semen collection:

Semen was collected in the morning from each cockerel once weekly by an abdominal massage method according to Lake and Stewart (1978) for five consecutive weeks as a semen collection period. Care was taken to avoid any contamination of semen with cloacae products and yellow and abnormal ejaculates were systematically discarded.

Only ejaculates with mass motility ≥70% on the day of collection were pooled on each collection day. Semen was evaluated for sperm cell concentration using a Neubauer hemacytometer, then semen was extended by the modified Beltsville Poultry Semen Extender (mBPSE) at a rate of 1 semen: 2 extender and divided into four parts; the 1st part was extended with control extender without any supplementation (T1). However, the 2nd, 3rd and 4th parts were supplemented with 0.2 (T2), 0.4 (T3) and 0.6 (T4) mM GSH ($C_{10}H_{17}N_3O_6S$), respectively, (S.d fine-Chem. Limited, India).

Semen extender supplemented with different GSH levels was stored in refrigerator at 5°C and evaluated after 0, 24, 48 and 72 h. Semen evaluation included PSM, SL, SA, acrosome status, and hypo-osmotic swelling test (HOST) of spermatozoa.

Semen evaluation:

The PSM was assessed according to Tabatabaei *et al.* (2009), while SL was determined by eosin and nigrosin stain as described by Lukaszewicz *et al*. (2008). The SA was determined during the examination of SL at a high power magnification (400 x).

As an alternative to evaluate the percentage of acrosomal abnormalities, staining procedure for fixed samples have been developed to distinguish which spermatozoa have retained or lost the acrosome to obtain percentage of spermatozoa with damaged acrosome (Al-Daraj, 2001).

The HOST as described by Lagares *et al.* (2000) was assessed to evaluate sperm membrane integrity. HOST-solution consisted of fructose (1.25%) and Na-citrate (2.9%) in distilled water (three times) to give osmolality at a level of 300 mOsm/l using osmometer (Osmett A, Model 5002, Fisher Svientific, Pittsbury, PA, USA). To reach osmolarity of 50 mOsm/l, distilled water was added using osmometer. About 0.1 ml of diluted semen was added to 0.9 ml of the HOST-solution on a glass tube and the mixture was examined at 37^{o}C after 30 min. Slides were stained by eosin-nigrosine stain and examined to determine number of sperm cells with curled tails using research microscope (x 400).

Fertility trial:

Artificial insemination was performed using syringes (1.0 ml) for the deposition of the semen according to Sadanand *et al*. (2004) to assess fertility rate. Hens from the same local breed were divided into 16 groups (7 hens for each treatment), including semen supplemented with 4 GSH level at 4 storage periods). Total of 256 eggs, 16 from each group were collected after 1 day post-insemination for a week, and incubated to evaluate the fertility rate (FR). Eggs were candled to identify the fertile eggs according to the method of Islam *et al.* (2002) as the following: FR = Fertilized eggs/ incubated eggs x100.

Statistical analyses:

Data obtained were statistically analyzed as a factorial design (4 GSH levels x 4 storage periods) by using analysis of variance ANOVA (SAS, 2001). The significant differences were set at 0.05 probability level and tested using the Duncan's procedure (Duncan, 1955). Data of fertility rates were analyzed using Chi-square.

RESULTS AND DISCUSSION

Interaction between GSH level and storage period:

No interaction effect between GSH level and storage period was observed in all studied traits ($P<0.05$). It is of interest to note that the data numerically indicated that semen extended with GSH at levels of 0.4 or 0.6 Mm had long storage period up to 24 h as compared with the control semen (<24 h). Moreover, semen supplemented with 0.2 mM GSH maintained all sperm characteristics studied for the longest storage period up to 48 h. However, unaccepted values of all sperm characteristics were obtained after 72 h of storage in all types of semen.

In accordance with the present results, Wang *et al*. (1997) observed an increase in ROS production in human semen stored at 4 °C by increasing storage time. Hydrogen peroxide (H_2O_2), as ROS has been shown to negatively affect sperm motility in mouse, human, bull, and rabbit (Alvarez and Storey, 1989).

In vitro storage of turkey sperm cells markedly affect the endogenous metabolism of the fatty acids of the membrane phospholipids and induce membrane destabilization, which was association with the decrease in quality of semen **(**Douard *et al.,* 2000). Under the condition of semen storage, supplementation of the extenders with different antioxidants can help to maintain motility and livability of spermatozoa (Bilodeau *et al*., 2001 and Foote *et al*., 2002).

Effect of GSH level:

Semen quality in terms of PSM, SL, CT, SA and AD were significantly (P<0.05) improved by adding GSH to semen extender at all levels as compared with free extender (control). Semen diluted with 0.2 mM GSH showed higher percentages of PSM, SL and CT, and lower percentages of SA and AD as compared with other groups (P<0.05). Semen without GSH (control) showed the poorest quality (Table 1). These results clearly indicate that addition of GSH to semen extender at a level of 0.2 mM had impact on all sperm characteristics studied, regardless storage period.

Table 1. Sperm characteristics of cockerel as affected by glutathione supplementation to semen extender stored at 5°C

GSH (mM)	Progressive motility (%)	Live sperm (%)	Abnormal sperm (%)	Acrosomal damage (%)	Curled tail (%)
0.0	33.00^{d}	39.20^{c}	18.60^{a}	20.40^{a}	31.65^{b}
0.2	44.50^{a}	54.00^{a}	14.80^{b}	16.35^{b}	42.00^{a}
0.4	40.00^{b}	45.95^{b}	16.70^{ab}	17.50^{ab}	38.10^{ab}
0.6	36.25^{c}	41.45^{bc}	17.75^{ab}	18.10^{ab}	36.15^{ab}
±SEM	0.848	0.881	0.558	0.462	0.849

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

In the current study, increasing GSH level above 0.2 mM improved most of semen quality traits. In addition, the best results was observed in semen supplementation of 0.2 mM GSH (P<0.05). In the literature we did not find publications referring to the effects of glutathione supplementation to semen extender on cockerel spermatozoa. However, in bull semen, adding GSH (0.4, 0.8 and 1.2 Mm) showed the best improvement at a level of 0.4 mM, but increasing GSH level up to 1.2 mM showed the lowest PSM and SL percentages as compared with those in the control semen store at 5°C. This may suggest that the toxic effect of this concentration affected negatively bull semen traits **(**El-Sherbieny *et al*., 2006). The effect of addition of natural antioxidants into semen diluents on the preservability of buffalo semen was studied by Raina *et al.* (2002). They found that sperm motility was significantly (P<0.01) affected by extender-antioxidant combination in semen stored at 4-7°C.

There are several reports about positive effects of GSH on sperm function. Addition of 0.5 -1.0 mM of GSH to diluents is sufficiently effective to protect the plasma membranes and maintain the percentage of bovine spermatozoa motility stored at 5 ºC (Triwulanningsih *et al.,* 2008). Also, in buffalo, El-Kon and Darwish (2011) indicating that addition of 0.50-1.00 mM of GSH to semen diluent improved quality of liquid semen stored up to 120 hours in terms of reducing DNA damage and improving the fertility.

During semen storage, oxidative damage to sperm resulting from ROS produced by the semen components. This finding is possibly one of the main reasons for the reduction in sperm motility and fertility in ram (Bucak and Tekin, 2007) and in bull (El-Sherbieny *et al*., 2006).

It is worth noting that GSH (0.1-1.0 mM) significantly inhibits ROS generation in chicken semen, but had no effect on post-thawed spermatozoa quality (Thananurak *et al.,* 2015). Also, others found no effect of GSH at 5 mM on progressive movement of post-thawed boars' sperm (Whitaker *et al.,* 2008) and on human sperm motility over a 4 h period (Donnelly *et al*., 1999). Similarly, no significant effect on semen quality of boar was found after addition of GSH to the thawing medium (Gadea *et al.,* 2005a).

In turkey, Long and Conn. (2012) found that additional of phosphatidylcholine as antioxidants to the semen reduced the harmful effects of lipid peroxidation during semen storage at 4°C. Also, administration of vitamins C, E, catalase (Amini *et al.,* 2015a, b) and oleic acid (Eslami *et al.,* 2016) to the rooster semen decreased the levels of malondialdehyde in seminal plasma and post-thawed of sperm.

Effect of storage period:

Data shown in Table (2) indicate that storage period had deleterious effect on all characteristics of cockerel spermatozoa. Percentages of PSM, SL and CT significantly (P<0.05) decreased, while those of SA and AD significantly (P<0.05) increased by advancing storage period. These findings were reported in turkey spermatozoa in semen preserved at cool temperature for 24 h (Sexton, 1986) and even in frozen semen by liquid nitrogen (Douard *et al*., 2000). In rooster, supplementation of oleate to semen extender caused an improve in total antioxidant activity concentration of seminal plasma and spermatozoa stored at 4°C for at 24 h and 48 h compared with control group (Eslami *et al.,* 2016).

Table 2. Sperm characteristics of cockerel as affected by storage period at 5°C

Storage period (h)	Progressive motility (%)	Live sperm (%)	Abnormal sperm (%)	Acrosomal damage (%)	Curled tail (%)
0	68.75[a]	75.95[a]	9.70[d]	10.55[d]	67.25[a]
24	37.25[b]	49.90[b]	14.45[c]	16.20[c]	39.85[b]
48	29.75[c]	35.10[c]	17.95[b]	1825[b]	24.70[c]
72	18.00[d]	19.65[d]	25.75[a]	27.35[a]	16.10[d]
±SEM	0.848	0.881	0.558	0.462	0.849

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

Fertility rate:

Results presented in Table (3) show that overall mean of fertility rate (FR) of eggs after performing the artificial insemination of hens was affected by GSH level and storage period (P<0.05). GSH supplementation significantly (P<0.05) increased FR. Semen supplemented with 0.2 mM GSH (74.5%) showed higher FR than those supplemented with 0.6 mM GSH or free semen (59.9 and 59.2%, respectively). However, FR of semen extended with 0.4 mM GSH did not differ significantly from other GSH levels or from the control semen. Also, FR showed gradual reduction by increasing storage time (P<0.05), being the highest at 0 h and the lowest in semen stored for 72 h (Table 3).

Moreover, FR was higher for semen supplemented with 0.2 mM GSH and stored at any period as compared with other semen treatments, being the highest for fresh semen (0 h) with 0.2 mM GSH (93.6%, Table 3). Such results of different types of semen indicate FR of about 57% for semen stored for 72 h in semen extended with 0.2 mM GSH. The GSH treatment and increasing storage time of chicken semen reduce the motility and may cause disruption in the membrane integrity of spermatozoa.

Table 3. Fertility rate (%) of eggs of hens inseminated by semen supplemented with different GSH levels and stored at various periods as affected by storage period

GSH level (mM)	Storage period (h)				Overall mean
	0	24	48	72	
0.0	90.9	61.6	50.3	33.9	59.2[b]
0.2	93.6	82.2	64.7	57.3	74.5[a]
0.4	91.8	71.5	54.6	55.6	68.4[ab]
0.6	91.1	53.9	52.8	41.91	59.9[b]
Overall mean	91.9[a]	67.3[b]	55.6[c]	47.2[d]	-

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

Fertility rates were analyzed by Chi-square student test.

It is generally assumed that these changes are detrimental and are associated with a loss of fertilizing capacity. No available information in the literature regarding the influence of GSH supplementation to cockerel semen stored at cool temperature on fertility rates. However, in general, FR is lower for stored than of fresh semen, after cervical insemination of rams (Maxwell and Watson, 1996). Mowever, addition of GSH has been shown to help to maintain sperm motility and improving sperm *in vitro* oocyte penetration ability (Gadea *et al.,* 2005b & 2011). In agreement with the obtained results, Amina (2002) reported that increasing storage time of diluted semen from local chicken breed for 0, 24, 48 and 72 h significantly (P<0.01) decreased FR by 5.12, 9.55 and 12.53%, respectively. In broiler breeder, supplementation of canthaxanthin or biotin in diets (Rosa *et al.,* 2012 and Daryabari *et al.,* 2015), improved the FR.

CONCLUSION

Semen supplemented with GSH was characterized by moderate resistance morphological defects, increases motility and livability of spermatozoa in preserved semen as well as fertility rates. Supplementation of GSH at a level of 0.2 mM to extender of cockerel semen stored at 5°C for up to 72 h is an appropriate level to improve the fertilizing ability of chicken spermatozoa.

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تأثير الجلوتاثيون المضاف الى مخفف السائل المنوى على خصائص السائل المنوى للديوك المحلية والمخزن على درجة حرارة ٥°م لفترات تخزين مختلفة

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تهدف هذه الدراسة الى تقييم تأثير اضافة الجلوتاثيون المخفض بمستويات صفر،٠.٢،٠.٤و٠.٦ملل مول لمخفف السائل المنوى المخزن لمدة صفر،٢٤، ٤٨ و٧٢ ساعة على درجة ٥°م على خصائص السائل المنوى ومعدل الخصوبة للديوك من سلالة انشاص. وقد اوضحت النتائج ان اضافة الجلوتاثيون بجميع المستويات لمخفف السائل المنوى حسنت من جودة السائل المنوى من حيث الحركة التقدمية ،الحيوية، الذيل الملتوى ، شواذ الحيوانات المنوية وتلف الأكروسوم مقارنة بالكنترول (سائل مخفف بدون جلوتاثيون). وقد اظهر اضافة تركيز ٠.٢ ملل مول جلوتاثيون الى المخفف أعلى معنوية بينما اظهرت مجموعة الكنترول (بدون جلوتاثيون) اقل معنوية بالنسبة لجودة للسائل المنوى. وكانت لفترة التخزين تأثيرات ضارة على جميع خصائص الحيوانات المنوية للديوك. وقد انخفضت معنويا النسبة المئوية للحركة التقدمية وحيوية الحيوانات المنوية بينما لوحظ زيادة معنوية فى شواذ الحيوانات المنوية وتلف الاكروسوم خلال فترات التخزين الطويلة. ولم يكن هناك فروق معنوية بين مستويات الجلوتاثيون ومدة التخزين ويعكس ذلك افضل النتائج لخصائص السائل المنوى مع كل فترات التخزين للسائل المنوى المخفف بمستوى ٠.٢ ملل مول من الجلوتاثيون. وجد ان معدل الخصوبة اعلى معنويا للسائل المنوى مع ٠.٢ ملل مول جلوتاثيون (٧٤.٥%) مقارنة بمستوى ٠.٦ ملل مول جلوتاثيون (٥٩.٩%) او السائل المنوى بدون جلوتاثيون (٥٩.٢ %). وبالرغم من ذلك لم يكن هناك فروق معنوية لمعدل خصوبة السائل المنوى المخفف بمستوى ٠.٤ ملل مول جلوتاثيون(٦٨.٤%) ومع المستويات الاخرى من الجلوتاثيون او حتى مجموعة الكنترول. وقد اظهر معدل الخصوبة انخفاض تدريجى مع فترات التخزين الطويلة فكانت اعلى معنويا مع صفر واقل عند٧٢ ساعة من تخزين السائل المنوى .وقد لوحظ ارتفاع معدل خصوبة السائل المنوى المخفف مع ٠.٢ ملل مول من الجلوتاثيون خلال جميع فترات التخزين بالمقارنة مع المامالات الاخرى من الجلوتاثيون. ونستخلص من هذه الدراسة ان اضافة ٠.٢ من الجلوتاثيون الى مخفف سائل منوى الديوك المخزن على درجة ٥°م لمدة تصل إلى ٧٢ ساعة هو المستوى المناسب لتحسين المقدرة الاخصابية للحيوانات المنوية للديوك من سلالة أنشاص المحلية.