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EFFECT OF THE REDUCED GLUTATHIONE ON THE BUFFALO-BULL SEMEN FREEZABILITY

BY

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ABSTRACT

This study aimed to investigate the effect of GSH on the freezability of buffalo-bull semen. It was carried out on 6 buffalo-bulls belonging to the farm of ARRI, Al Haram, Giza, Egypt during the period from September 2012 to May 2013. Semen was collected twice weekly, evaluated, diluted with TEYG extender supplemented with 0, 1, 5 and 10 mM of GSH then preserved in LN. Post-thawing motility, viability index, acrosomal integrity, enzyme leakage and LPO were assessed. The results showed that the highest post-thawing motility (65.00 ± 2.89 %), viability index (159.17 ± 7.95) and the lowest acrosomal abnormalities (10.33 ± 2.40 %) were recorded with 5 mM GSH. Moreover addition of 5 mM GSH to semen extenders gave the highest TAC (0.83 ± 0.04 µmol/ml) and the lowest activities of AST (35.67 ± 4.33 IU/L), ALT (14.67 ± 2.40 IU/L) and ALP (11.33 ± 2.40 IU/L) enzymes and MDA (6.50 ± 1.67 nmol/ml). In conclusion, it is advised to supplement the buffalo-bull semen extenders with 5 mM GSH to improve freezability.

Key words: Glutathione, cryopreservation, buffalo-bull, semen.

INTRODUCTION

Water buffaloes (Bubalus bubalis) are considered one of the most important farm animals kept for dual purposes (milk/meat production). In Egypt, there are about 5.317 million heads, producing 44 % and 18 % of total milk and meat production respectively **(FAOSTAT, 2013).**

Despite their importance for milk and meat production, they have not received sufficient attention regarding the improvement of reproductive performance **(Vale, 1997)**.

Artificial insemination (AI) has been extensively used by developed countries for rapid genetic improvement through exploiting the germplasm of the superior males. The benefits of AI technique can be fully achieved by successful freezing-thawing of semen without compromising its fertility and so far, this has been met with a little success in buffalo **(Andrabi, 2009).**

Oxidative stress (OS) was the major limiting factor in buffalo semen cryopreservation. Cryopreservation not only increases reactive oxygen species (ROS) production, but also decreases the antioxidant potential of semen **(Bilodeau et al., 2000)**.

OS could deteriorate the fertility of buffalo bull semen probably by the impairment of sperm motility, viability, plasma membrane, acrosomal and DNA integrity **(El-Sisy et al., 2007).**

Due to the endogenous antioxidant defense system of mammalian semen is not enough to protect the spermatozoa during cryopreservation against OS, supplementation of semen extenders with exogenous antioxidants is recommended to reduce the cryodamage of spermatozoa **(Ansari and Shah, 2011)**.

Glutathione (GSH) is a tripeptide (γ-glutamyl-cysteinyl-glycine), naturally presents in buffalo semen and has been recognized as an essential non-enzymatic antioxidant. Therefore, it was founded that fortification of semen extender with GSH improved the post-thawing quality **(Gadea et al., 2005)**.

The present study aimed to investigate the effect of supplementation of extenders with different concentrations of GSH on the freezability of buffalo semen.

MATERIALS AND METHODS

1. Experimental animals:

This study was carried out on 6 buffalo bulls of 4-6 years old and 400-600 kg weight belonging to the farm of Animal Reproduction Research Institute (ARRI), Al-Haram, Giza, Egypt. They were maintained under optimum nutritional and managemental practices as per the standard criteria fixed for maintenance of breeding bulls in bull stations.

2. Experimental design:

2.1. Semen collection, processing and preservation:

Twice a week, 2 successive ejaculates were collected from each buffalo-bull by Artificial Vagina (AV) method as described by **Abd El-Malak (1989)**. Immediately after semen collection, each ejaculate was evaluated according to **Sansone et al. (2000)**. Only semen samples of at least 70 % individual motility and 800.00 x 10^6 sperm cells / ml were pooled together for semen processing and preservation.

Pooled semen samples were divided into 4 equal portions and diluted (1:8) with Tris-Egg Yolk-Glucose (TEYG) extender **(Santiani et al., 2005)** supplemented with different concentrations of GSH (0, 1, 5 and 10 mM) at 30°C. The diluted semen was then preserved by liquid nitrogen (LN) as per method described by **Mohammed et al. (1998)**.

2.2. Evaluation of semen freezability:

The straws were stored in LN tank for at least 24 hour before evaluation. Randomly selected 3 frozen straws from each treatment were removed and thawed in a water bath at 37°C for 30 seconds and the frozen-thawed semen was then microscopically and biochemically evaluated.

2.2.1. Microscopical evaluation:

Included assessment of post-thawing motility **(Zemjanis, 1970)**, viability index **(Milovanov, 1962)** and acrosomal integrity **(Chinoy et al., 1992)**

2.2.2. Biochemical evaluation:

Included estimation of activities of aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and alkaline phosphatase (ALP) enzymes **(Kind and King, 1954; Reitman and Frankel, 1957)**, total antioxidant capacity (TAC) **(Hirai et al., 2011)** and malondialdehyde (MDA) using thiobarbituric acid (TBA) as per the method described by **Buege and Aust (1978)** and modified by **Suleiman et al. (1996)**.

2.3. Statistical analysis:

Data were collected, organized, summarized and then statically analyzed by using statistical package SPSS (ver. 20). One way analysis of variance (ANOVA) was used to test variance between different groups.

RESULTS

As shown in table 1 there was a significant variation ($p<0.05$) in post-thawing motility %, viability index and acrosomal defects % when buffalo-bull semen was diluted with TEYG extender supplemented by different concentration (0, 1, 5 and 10 mM) of GSH. The highest post-thawing motility (65.00 ± 2.89%), viability index (159.17 ± 7.95) and the lowest acrosomal defects (10.33 ± 2.40%) were recorded with 5 mM GSH.

As shown in table 2 there was a significant variation ($p<0.05$) in the activities of AST, ALT and ALP enzymes, TAC and MDA level when buffalo-bull semen was diluted with TEYG extender supplemented by different concentration (0, 1, 5 and 10 mM) of GSH. The highest TAC (0.83 ± 0.04 μmol/ml) and the lowest activities of AST (35.67 ± 4.33 IU/L), ALT (14.67 ± 2.40 IU/L) and ALP (11.33 ± 2.40 IU/L) enzymes and MDA level (6.50 ± 1.67 nmol/ml) were recorded with 5 mM GSH.

Table 1: Effect of addition GSH to extender on microscopical parameters of frozen-thawed buffalo-bull semen

Parameters / GSH	Dilution motility%	Post-thawing motility%	Viability index	Acrosmal defects%
0 Mm (Control)	**75.00 ± 2.89 [a]**	**31.67 ± 7.26 [b]**	**64.17 ± 10.83 [b]**	**33.00 ± 3.21 [a]**
1 mM	**78.33 ± 1.67 [a]**	**40.00 ± 5.77 [b]**	**88.33 ± 19.65 [b]**	**19.33 ± 4.37 [bc]**
5 mM	**76.67 ± 4.41 [a]**	**65.00 ± 2.89 [a]**	**159.17 ± 7.95 [a]**	**10.33 ± 2.40 [c]**
10 mM	**63.33 ± 6.01 [a]**	**36.67 ± 4.41 [b]**	**71.67 ± 15.83 [b]**	**21.67 ± 3.48 [b]**

Values are expressed as means ± SE

Means with different superscript letters significantly differ at least ($p<0.05$)

**GSH = reduced glutathione*

Table 2: Effect of addition GSH to extender on microscopical parameters of frozen-thawed buffalo-bull semen

Parameters / GSH	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	TAC (µmol/ml)	MDA (nmol/ml)
0 Mm (Control)	107.00 ± 9.29 [a]	67.00 ± 4.36 [a]	49.67 ± 2.73 [a]	0.17 ± 0.09 [b]	30.05 ± 3.03 [a]
1 mM	78.33 ± 7.06 [b]	61.33 ± 6.06 [a]	29.33 ± 1.45 [b]	0.26 ± 0.05 [b]	22.77 ± 1.57 [b]
5 mM	35.67 ± 4.33 [c]	14.67 ± 2.40 [b]	11.33 ± 2.40 [c]	0.83 ± 0.04 [a]	6.50 ± 1.67 [c]
10 mM	83.67 ± 4.18 [b]	55.67 ± 4.41 [a]	35.67 ± 4.91 [b]	0.28 ± 0.09 [b]	20.01 ± 0.91 [b]

Values are expressed as means ± SE

Means with different superscript letters significantly differ at least ($p<0.05$)

**AST= aspartate-aminotransferase, * ALT= alanine-aminotransferase,*
**ALP= alkaline phosphatase,*
**TAC= total antioxidant capacity, * MDA = malondialdehyde*

DISCUSSION

The high concentration of poly unsaturated fatty acids (PUFAs) in the plasma membrane of buffalo spermatozoa renders them more susceptible to lipid peroxidation (LPO) due to OS induced by cryopreservation **(Aitken et al., 1998)**. LPO in turn causes a reduction in motility, viability and integrity of plasma membrane, acrosome and DNA of frozen-thawed buffalo spermatozoa **(Anzar et al., 2010; Kumar et al., 2011).**

This may explain the results of the present which showed that supplementation of extender with improved the post-thawing motility, viability and integrity of plasma membrane, acrosome and DNA of bubaline semen. These results are in agreement with previous studies on bovine **(Gadea et al., 2005)**, ovine **(Bucak and Tekin, 2008)**, caprine **(Sinha et al., 1996)**and swine **(Funahashi and Sano, 2005)** semen.

It is known that, freeze-thawing cycle could reduce the motility and viability of buffalo spermatozoa by more than 50% due to overproduction of ROS specially H_2O_2 **(Garg et al., 2009a).** GSH acts as a cofactor of glutathione peroxidase (GPx) enzyme which not only scavenges hydro peroxides (H_2O_2), but also scavenges lipid peroxides (ROOH) **(Halliwell, 1989).**

In the current study, addition of GSH to extender before freezing resulted in a higher post-thawing motility and viability in a dose dependent manner. These results were previously reported by **Ansari et al. (2010)** and **Ansari et al. (2011)** who clarified that improvement of the post-thawing motility and viability of buffalo spermatozoa was due to the protective action of GSH on the motility apparatus during cryopreservation.

Intactness of acrosome is critical for acrosomal reaction and development of embryo following completion of fertilization process. The freeze-thawing cycle decreased the population of sperm with intact acrosomes **(Rasul et al., 2001)**. Naturally occurring antioxidants in semen protect the acrosomal integrity of the spermatozoa by reducing levels of ROS molecules and lipid peroxidation of cell membrane **(Cotran et al., 1989)**. **Bilodeau et al. (2000)** reported that the activity of GSH in frozen-thawed semen decreased by 50% compared with fresh semen. Therefore, it was found that fortification of semen extenders with GSH protected acrosmal integrity. This is consistent with results of **Sinha et al. (1996)** and **Perumal et al. (2011)**.

Besides the impairment of motility, viability and acrosomal integrity, LPO could cause disintegration of cell membrane leading to leakage of intracellular enzymes like AST, ALT and ALP **(Rasul et al., 2001)**. MDA is a stable by-product of LPO. Therefore, measurement of MDA is widely used as an indicator of LPO level in a variety of cell types, including spermatozoa **(Agarwal and Prabakaran, 2005)**.

The current results demonstrated that the presence of GSH in the freezing extender not only decreased the leakage of AST, ALT and ALP enzymes and MDA production, but also increase the total antioxidant capacity (TAC) in frozen-thawed buffalo spermatozoa. These results corroborate the hypothesis that one of the most beneficial effects of GSH during the cryopreservation is reduction of the LPO, throughout scavenging of lipid peroxides. These results are in the line with those of **Garg et al. (2009b)**, **Kadirvel et al. (2009)** and **Kumar and Atreja (2012)**. In conclusion, addition of 5 mM GSH to semen extender improved freezability of buffalo-bull spermatozoa.

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الملخص العربى

دراسة تاثير الجلوتاثيون المختزل على قابلية حيامن طلائق الجاموس للتجميد

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تهدف هذه الدراسة الى الوقوف على مدى تاثير اضافة الجلوتاثيون المختزل على قابلية حيامن طلائق الجاموس للتجميد. اجريت الدراسة على ٦ طلائق جاموس فى مزرعة معمل بحوث وتناسليات الهرم بالجيزة خلال الفترة من سبتمر ٢٠١٢ حتى مايو ٢٠١٣. تم تجميع عينات السائل المنوى من طلائق الجاموس مرتين اسبوعيا وبعد تقييمها معمليا تم تمديدها فى ممد التريس فقط (المجموعة الضابطة) او المضاف اليه تركيزات مختلفة من الجلوتاثيون المختزل (١ ، ٥ ،١٠ ملي مول) وبعد تبريدها وتجميدها فى النيتروجين السائل تم تقيمها بعد الاسالة من حيث نسبة الحركة والحيوية وسلامة غشاء القلنسوة وتسرب الانزيمات ومستوى الاكسدة الكلية ومعدل اكسدة الدهون. وقد اوضحت نتائج الدراسة الحالية بان اضافة الجلوتاثيون المختزل بتركيز ٥ ملي مول الى ممدد التريس حقق اعلى نسبة فى الحركة (٦٥,٠٠±٢,٨٩٪) والحيوية (١٥٩,١٧± ٧,٩٥) واقل نسبة فى تشوهات غشاء القلنسوة (١٠,٣٣± ٢,٤٠٪) كما انه حقق اعلى مستوى فى مضادات الاكسدة الكلية (٠,٨٣± ٠,٠٤ ميكرو مول / ملى) واقل مستوى من حيث تسرب الانزيمات (٣٥,٦٧±٤,٣٣ و ١٤,٦٧±٢,٤٠ و ١١,٣٣±٢,٤٠ وحدة دولية / لتر) وايضا اقل معدل لاكسدة الدهون (٦,٥٠-١,٦٧ نانو مول / ملى). نستنتج مما سبق انه ينصح باضافة الجلوتاثيون بتركيز ٥ ملى مول الى ممدد السائل المنوى من اجل الحصول على اعلى قابلية لحيامن طلائق الجاموس للتجميد.