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EFFECT OF DIFFERENT TYPES AND LEVELS OF ENZYMATIC ANTIOXIDANTS ON SOME CHARACTERISTICS AND FERTILITY OF BUFFALO SPERMATOZOA OF FROZEN SEMEN

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SUMMARY

The effects of different types and levels of enzymatic antioxidants including catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD) added to Tris-egg yolk extender (TEYE) on some characteristics and fertility of buffalo spermatozoa in frozen semen. Semen was collected twice weekly by artificial vagina from five sexually mature Egyptian buffalo bulls and held in a water bath at 35-37°C. Ejaculates with ≥70% mass motility were pooled for each collection day for 10 wk (100 pooled ejaculates). Pooled semen was diluted (1:20) and divided into 7 comparable portions and diluted (1:20) with TEYE either without antioxidant (control) or supplemented with 200 IU CAT, 400 IU CAT, 0.4 mM GSH, 0.8 mM GSH,150 IU SOD or 300 IU SOD (treated groups), respectively. Semen diluted with different types of TEYE was equilibrated at 5°C for 4 h and processed for freezing using 0.25 ml French straws and stored at –196oC for at least one month. Percentages of progressive motility ((SM), livability (SL) and acrosome damage (AD) of sperm cells were determined in post- diluted, -equilibrated and -thawed semen. Results revealed insignificant differences for SM, SL and AD in post-diluted semen among all tested extenders. In post-equilibrated and post–thawed semen adding antioxidants to TEYE extender significantly improved SM, SL and AD (P<0.05) than control. In post-equilibrated semen no significant differences were found in either SM or SL among the 6 treated groups, while in post-thawed semen level of antioxidant was significantly (P<0.05) effective in this concept, where 400 IU CAT, 0.4 mM GSH and 300 IU SOD were superior than 200 IU CAT, 0.8 mM GSH and 150 IU SOD, respectively. Conception rate was the highest (75%, P<0.05) for buffalo cows inseminated with semen supplemented with 400 IU CAT, 0.4 GSH or 300 SOD and the least CR (58.3%) was recorded for control semen. In conclusion, high level of catalase (400 vs. 200 IU) or superoxide dismutase (300 vs. 150 IU) and low level of reduced glotathion (0.4 vs. 0.8 mM) as enzymatic antioxidants showed beneficial effects on some characteristics and fertilizing capacity of buffalo spermatozoa of frozen semen.

Keywords: Buffalo semen, enzymatic antioxidants, fertilizing capacity

INTRODUCTION

Buffalo spermatozoa contain comparatively more unsaturated fatty acids than in other species, like arachidonic and decosahexaenoic acids, which make them more vulnerable to lipid peroxidation (Sreejith *et al*., 2005).

During various processing procedures of freezing semen, sperm cells used in AI are exposed to oxygen and visible light radiation, which could lead to lipid peroxidation and formation of ROS, which negatively affect sperm cell motility and genomic integrity (Bilodeau *et al*., 2000). The endogenous antioxidative capacity of semen may be insufficient during storage or dilution (Maxwell and Salamon, 1993). A significant reduction in the level of spermatozoa antioxidant been reported as one of the causes of the enhanced susceptibility of these cells to peroxidative injuries after cryopreservation (Bilodeau, *et al*., 2000). So, adding several types of antioxidant enzymes active in scavenging ROS, which could help to maintain survival and motility of spermatozoa (Bilodeau *et al.,* 2000 and Foote *et al.,* 2002).

The lipid peroxidation cascade is initiated when spermatozoa are attacked by ROS, which results in a loss of polyunsaturated fatty acids from the plasma membrane and a corresponding decline in the survival and fertilizing ability of these spermatozoa (Aitken, 1995). Sperm oxidative damage is the result of an improper balance between ROS generation and scavenging activities. *In vitro* studies suggested that the addition of CAT (Abdel-Khalek *et al.,* 2009), GSH (Ahmed, 2008) or SOD (El-Nagar, 2008) to diluted semen could improve the motility and survival of buffalo bull spermatozoa in frozen semen as compared to controls. However, there is no information on the comparison of the beneficial effects of these enzymes on buffalo sperm function and fertility. Therefore, the current work aimed to compare the effects of adding different types and levels of enzymatic antioxidants (200 and 400 IU of CAT), 0.4 and 0.8 mM of GSH or 150 and 300 IU of SOD to Tris-egg yolk extender on some characteristics and fertility of buffalo spermatozoa of frozen semen.

MATERIALS AND METHODS

Semen collection:

Five sexually mature buffalo bulls aged 4-7 years and weighed 548.6±11.24 were used for semen collection. All bulls were healthy and clinically free of external and internal parasites. Palpation of the external genitalia showed that they were typically normal. Semen was collected during autumn, 2009 by artificial vagina set up at optimal conditions to induce a good ejaculatory thrust. On day of collection, one false mount had been allowed before collection of the experimental ejaculates. Semen was collected twice weekly from each bull, where immediately after collection, ejaculates were held in a water bath at 35-37℃, before being transferred to the laboratory. Ejaculates having good mass motility (>70%) were pooled for each collection day.

Semen extension:

Total of 100 ejaculates were obtained during a collection period of 10 weeks. The main extender used for semen dilution was Tris-egg yolk extender (TEYE) containing 3.025 g Tris (hydroxymethyl amino methane, 1.675 g citric acid, 0.75 g glucose, 15 ml egg yolk, 7 ml glycerol, 0.005 g streptomycin, 0.25 g lincospectin and completed with bi-distilled water up to 100 ml. Total of 7 extenders, 6 of them with 3 types of antioxidant with two levels including CAT (200 and 400 IU), GSH (0.4 and 0.8 mM) and SOD (150 and 300 IU) were compared to extender without additive (control).The dilution rate was 1:20. The Tris-egg yolk extender was gently mixed and warmed up to 37oC in a water bath 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.

Freezing processes:

For gradual cooling, straws were kept in iced water both to keep its temperature at 5°C, while semen packed in straws was placed in a cooled ice chest. Post-equilibration, the extended semen was loaded in 0.25 ml French straws using a semen filling machine. 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. Each straw was titled for each supplementation and collection week then stored for at least one month.

Semen evaluation:

Percentages of SM (Amman and Hammerstedt, 1980), SL (Hancock, 1951) and AD (Watson, 1975) were determined in post-diluted and post-equilibrated semen during freezing process as well as in post-thawed semen (20 straws for each extender). For thawing, straws were dipped into a water bath at 37 - 38°C for 30 seconds.

Conception rate (%):

Total of 84 sexually mature buffalo cows aged 3.5-8 years, weighed 520.14±18.47 kg and between 2 and 5 parities were randomly divided into 7 groups (12 animals in each group) according to age, LBW and parity. Each buffalo cow in heat was artificially inseminated with semen containing different antioxidant supplements. Inseminated buffalo cows were diagnosed for pregnancy on day 40 post-insemination for determination of conception rate.

Statistical analysis:

Data obtained in this study were statistically analyzed using computer program of SAS (2004). One way ANOVA was used to perform the effects of type and level of some antioxidants. Multiple rang test (Duncan, 1955) was employed to test statistical differences among means at $P<0.05$. However, conception rate was analyzed using Chi square analysis. The percentage values were subjected to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from transformed values to percentages.

RESULTS AND DISCUSSION

Sperm progressive motility(SM):

Results shown in Table (1) revealed insignificant effect of antioxidant supplementation on SM in post-diluted semen. This indicated the absence of any effect of either the source or the level of antioxidant on SM after dilution of buffalo semen (Abdel-Khalek *et al.*, 2009**,** El-Nagar, 2008 and El-Seify *et al.*, 2009). Table (1) further shows that all antioxidant supplements significantly ($P<0.05$) increased SM in post-equilibrated and post–thawed semen than the control. Moreover, no significant differences were found among the various antioxidant supplements in this concept in post-equilibrated semen and the highest SM was recorded for 400 IU CAT (65.6%) and the lowest one for 0.4 mM GSH (62.5%). On the contrary, SM in post-

thawed semen significantly differed (P<0.05) among antioxidant supplemented levels, being significantly (P<0.05) higher 57.9, 54.5 and 56.3 % for 400 IU CAT, 0.4 IU GSH and 300 mM SOD than 47.3, 47.3 and 45.5 % for 200 IU CAT, 0.8 IU GSH and 150 mM SOD, respectively.

Table 1. Effect of antioxidant supplementation on percentages of sperm progressive motility in post-diluted, post-equilibrated and post-thawed of Buffalo semen

Extenders	Sperm progressive motility (%) in semen		
	Post-dilution	Post-equilibration	Post-thawing
Control	71.8	53.6b	34.3c
+200 IU Catalase	72.3	63.1a	47.3b
+400 IU Catalase	72.3	65.6a	57.9a
+0.4 mM GSH1	70.6	62.5a	54.5a
+0.8 mM GSH1	70.5	62.6a	47.3b
+150 IU SOD2	70.6	62.6a	45.5b
+300 IU SOD2	71.8	65.1a	56.3a
±SEM	1.12	1.41	1.87

a,b and c: Means within the same column with different superscripts are significantly different at P<0.05.
1GSH= Reduced glutathione 2SOD=Superoxide dismutase

These results are in agreement with those previously reported by El-Gaafary *et al*. (1990) and Fatouh and Abdou (1991) who observed an increase in sperm motility of bull semen supplemented with different CAT levels to egg yolk extender and semen stored at 5°C. El-Seify *et al.* (2009) and Abdel-Khalek *et al*. (2009) also found that buffalo semen extended with extender containing high CAT levels (400 and 1000 IU) resulted in higher viability indices than both low CAT level as well as the control un supplemented semen. Such effect was associated with the lowest energy utilization in extender containing high CAT level (1000 IU), whereas the highest utilization was observed in extender containing low level of CAT (125 IU).

It is worthy noting that low GSH level (0.4 mM) and high SOD level (300 IU) had benefits on sperm motility in post-thawed semen as compared to high GSH level (0.8 mM) and low SOD level (150 IU) and both 0.4 mM GSH and 150 IU SOD levels did not differ significantly than 400 IU of CAT. In the same line, Ahmed (2008) found that increasing level of GSH more than 0.4 mM did not affect significantly sperm motility in post-thawed buffalo semen. Also, the observed improvement in sperm motility was attributed to that SOD was able to attenuate the inhibition of sperm motility induced by a high concentration of ROS (Mammoto *et al.,* 1996). In addition, inclusion of SOD significantly delayed the destabilization of sperm plasma membrane associated with frozen storage of sperm cells (Maxwell and Stojanov, 1996).

Sperm livability (SL):

Results presented in Table (2) indicate insignificant effect of antioxidant supplementation on SL in post-diluted semen. Similar results were obtained on the effect of SOD (El-Nagar, 2008), GSH (Ahmed, 2008) or CAT (El-Seify *et al*., 2009)

In both post-equilibrated and post-thawed semen, antioxidant supplementation significantly (P<0.05) improved SL in comparing with the control, however, no significant differences were found due to either type or level of supplemented antioxidant in this concept. However, in post-thawed semen, the SL values were significantly affected (P<0.05) by level of antioxidant. Values of SL obtained by 400 IU CAT, 0.4 IU GSH and 300 mM SOD supplemented extenders were significantly superior (60.5, 62.4 and 59.5 % versus 50.1, 55.8 and 50.1 %) obtained by 200 IU CAT, 0.8 IU GSH and 150 mM SOD, respectively (Table 2). This finding indicates the impact of 0.4 mM, 300 IU SOD or 400 IU CAT on sperm livability in frozen buffalo semen.

Table 2. Effect of antioxidant supplementation on percentages of sperm livability in post-diluted, post-equilibrated and post-thawed of Buffalo semen

Extenders	Sperm livability (%) in semen		
	Post-dilution	Post-equilibration	Post-thawing
Control	72.2	55.1b	36.1c
+200 IU Catalase	73.7	66.5a	50.1b
+400 IU Catalase	73.8	68.3a	60.5a
+0.4 mM GSH1	73.0	69.7a	62.4a
+0.8 mM GSH1	73.6	69.6a	55.8b
+150 IU SOD2	73.4	69.0a	50.1b
+300 IU SOD2	73.2	69.2a	59.5a
±SEM	0.95	1.24	1.61

a,b and c: Means in the same column with different superscripts significantly differed, P<0.05.
1GSH= Reduced glutathione 2SOD=Superoxide dismutase

The addition of CAT was found to have positive effects in maintaining the bull SL in an egg yolk extender but not in milk extender (Foote *et al*., 2002). This lake may be due to the high content of antioxidants in milk casein (Taylor and Richardson, 1980). El-Seify *et al.* (2009) also observed increasing SL in post-equilibrated and post–thawed buffalo semen with the high SOD and high CAT doses than low doses. Ahmed (2008) and Abdel-Khalek *et al*. (2009) also reported beneficial effect of a low GSH dose than a high one on SL in post-thawed buffalo semen.

Acrosome status:

Data in Table (3) show insignificant effect of antioxidant supplementation on percentage of sperm AD in post-diluted semen, which agrees with results reported by El-Nagar (2008), Ahmed (2008) and El-Seify *et al*. (2009). However, supplementation of 400 IU of CAT, 0.4 mM of GSH or 300 IU of SOD to TEYE significantly (P<0.05) induced higher reduction in AD percentage in post-equilibrated and post-thawed semen, being the best for CAT supplementation. Data available in the literature, showed that supplementation of TEYE with CAT (Abdel-Khalek *et al*. 2009 and El-Seify *et al.,* 2009), SOD (El-Nagar, 2008 and El-Seify *et al.,* 2009) or GSH (Ahmed, 2008) have positive effects in maintaining higher percentage of sperm cell with intact acrosome in post-thawed buffalo semen. Moreover, Maxwell and Stojanove (1996) showed that CAT improved acrosome integrity of ram spermatozoa during liquid storage.

Table 3. Effect of antioxidant supplementation on spermatozoa with damaged acrosome% in post-diluted, post-equilibrated and post-thawed semen

Extenders	Sperm with damage acrosome (%) in semen		
	Post-dilution	Post-equilibration	Post-thawing
Control	24.8	41.0a	58.7a
+ 200 IU Catalase	26.3	32.0bc	44.5bc
+ 400 IU Catalase	22.7	28.5c	33.2e
+ 0.4 mM GSH1	23.6	28.8c	36.3de
+ 0.8 mM GSH1	22.8	29.8c	42.8c
+ 150 IU SOD2	25.5	34.8b	48.1b
+ 300 IU SOD2	23.7	29.0c	37.2d
±SEM	1.59	1.51	1.44

a,b…..d: Means in the same column with different superscripts significantly differed, P<0.05.
1GSH= Reduced glutathione 2SOD=Superoxide dismutase

Conception rate (CR):

Data in Table (4) recorded significantly higher CR 75% (9/12), (P<0.05) for buffalo cows inseminated by semen diluted by 400 IU CAT, 0.4 mM GSH or 300 IU SOD supplemented TEYE than 66.7% (8/12) for those inseminated by semen extended with 200 IU CAT or 150 IU SOD and 58.3%, (7/12) for the control cows inseminated with semen having no antioxidants supplement. These finding agree with that reported by Abdel-Khalek *et al*. (2009) on the effect of CAT and GSH on CR of buffalo cows. In addition, Maxwell and Stojanov (1996) detected that pre-storage fortification of semen extender with SOD significantly enhanced the viability and fertilizing ability of stored spermatozoa.

Table 4. Effect of antioxidant supplementation on conception rate of buffalo cows inseminated with tested extenders

Extenders	Sperm with intact acrosome damage (%) in semen		
	Inseminated animals	Conceived Animals	Conception Rate (%)
Control	12	7	58.3b
+200 IU Catalase	12	8	66.7ab
+400 IU Catalase	12	9	75.0a
+0.4 mM GSH1	12	9	75.0a
+0.8 mM GSH1	12	7	58.3b
+150 IU SOD2	12	8	66.7ab
+300 IU SOD2	12	9	75.0a

a and b: Means in the same column with different superscripts significantly differed, P<0.05.
1GSH= Reduced glutathione 2SOD=Superoxide dismutase

In general, low concentration of reactive oxygen species (ROS), including H2O2, can be required to promote capacitation, leading to the acrosome reaction of spermatozoa (Griveau *et al*., 1994). However, substantial accumulations of ROS can negatively affect reproductive function and affect sperm cells in a variety of ways (Aitken *et al.*, 1998 and Shen *et al*., 1999), with peroxidative damage to sperm DNA and the plasma membrane. The increase in oxidative modification of proteins with aging has been associated with oxygen-free radical generating systems. This increase

can be inhibited by CAT (Stadtman, 1992) SOD (Maxwell and Stojanove 1996) or GSH (El-Nenaey *et al.,* 2006).

Protection of sperm against H2O2 by the addition of catalase may be useful, particularly because bull semen contains little amount of catalase. In accordance with the present results, studies on dromedary camel semen showed the highest percentages of motility, live sperm and normal spermatozoa in semen diluted with extenders supplemented with 500 IU/ml of catalase (Medan *et al*., 2008). Also, Roca *et al*. (2005) found that addition of catalase to semen extender improved post-thaw sperm viability and fertility in boars. In addition, Sikka (2004) reported that catalase is a potent antioxidant and a very potent and efficient endogenous radical scavenger. It reacts with the highly toxic hydroxyl radical and provides protection against oxidative damage to biomolecules. De Lamirande and Gagnon (1995) stated that in vitro treatment of spermatozoa with SOD significantly inhibited the destabilization of periacrosomal sperm plasma membrane associated with sperm capacitation and acrosome reaction.

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تأثير أنواع ومستويات مختلفة من الإنزيمات المضادة للأكسدة علي بعض صفات والقدرة الإخصابية للسائل المنوي المجمد لطلائق الجاموس

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تمت هذه الدراسة بهدف التعرف على تأثير بعض مضادات الاكسدة على صفات السائل المنوى المجمد ومقدرتة الاخصابية لطلائق الجاموس تم جمع السائل المنوي باستخدام المهبل الصناعي من ٥ طلائق جاموسى عمر ٤-٧ سنوات مرتين أسبوعيا حيث وضعت العينات في حمام مائي على درجة حرارة ٣٧ مْ. تم خلط العينات التي تزيد حيويتها عن ٧٠% في كل يوم جمع و لمدة ١٠ أسابيع (١٠٠ قذفة) حيث تم تقسيم العينات بعد خلطها إلى ٧ أجزاء متماثلة. الجزء الأول تم تخفيفه باستخدام مخفف الترس مع صفار البيض دون إضافة مضادات الأكسدة (مقارنة) بينما الأجزاء الست الأخرى أضيف إليها على الترتيب إنزيم الكتاليز بمستويين ٢٠٠ , ٤٠٠ وحدة دولية ، SUPEROXIDE DISMUTASE بمستويين ١٥٠, ٣٠٠ وحدة دولية أو الجلوتاثيون بمستويين ٠.٤ ، ٠.٨ مليمول.. كانت نسبة التخفيف ١ :٢٠، فترة الأتزان لمدة ٤ ساعات على درجة ٥مْ ثم تعبئتها في قصيبات سعتها ٠.٢٥ مل وتجميدها وتخزينها في النتروجين السائل على درجة حرارة -١٩٦° م لمدة شهر على الأقل.بعد ذلك تمت إسالة العينات المجمدة بوضعها في حمام مائي لمدة ٣٠ ثانية على درجة حرارة ٣٨° م . تم تقدير النسب المئوية للحركة التقدمية للحيوانات المنوية ، حيوية وسلامة الأكروسوم بعد التخفيف، فترة الأتزان والتجميد والإسالة . أشارت النتائج إلى تفوق صفات السائل المنوى المجمد المضاف له مضادات الاكسدة عن المجموعة المقارنة . إضافة مضادات الاكسدة سببت تحسن معنوى على مستوى ٥% بعد فترة الاتزان وبعد فترة الاسالة فى كل من الحركة التقدمية وحيوية الحيوانات المنوية الحية مع نقص معنوى فى نسبة الاكروسوم الغير سليم عن مجموعة المقارنة. كما لم تلاحظ فروق معنوية فى هذا الصدد بعد فترة الإتزان بين أنواع مضادات الأكسدة باستثناء اضافة ١٥٠ وحدة دولية من DOS التى أعطت نسبة معنوية أعلى لتلف الأكروسوم (٣٤.٨%) مقارنة بمضادات الأكسدة الأخرى (٣٢.٠ - ٢٩.٨%) مقابل ٤١.% لمجموعة المقارنة, ارتفع معدل الاخصاب معنويا فى اناث الجاموس (٧٥ %) الملقحة بالسائل المنوى المجمد المضاف الية اما٤٠٠ وحدة دولية من الكتاليز أو ٠.٤ مليمول من الجلوتاثيون أو ٣٠٠ وحدة دولية من DOS مقابل (٥٨.٣%) لمجموعة المقارنة يتضح من هذه الدراسة أن اضافة أى من الكتاليز (٤٠٠ حدة دولية) أو SUPEROXIDE DISMUTASE (٣٠٠ وحدة دولية) أو الجلوتاثيون ٠.٤ مليمول) كمضادات للاكسدة كان لها تاثير فعال على المقدرة التخزينية والقدرة الإخصابية للسائل المنوى الجاموسى