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Addition of Glutathione to Semen Extender During Sperm Sorting can Improve *in vitro* Embryonic Development and *in vivo* Fertility in Buffalo

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Abstract: This study evaluated the effects of Glutathione (GSH) supplemented in the semen extender during the buffalo sperm sorting process on sperm quality, embryonic development after IVF and pregnancy rate after AI. The percentage of sperm motility, plasma membrane integrity and DNA fragmentation were detected by flow cytometry or by microscopy in stained, sorted and frozen semen treated with or without 0.75 mM GSH during the flow sorting procedure. The cleavage and blastocyst rates were examined at day 2 and 6-8 after IVF with frozen semen treated with or without 0.75 mM GSH. Pregnancy diagnosis was determined by transrectal palpation at 90 day after AI with frozen semen treated with or without 0.75 mM GSH. The percentage of sperm with Progressive Motility (MP, %) was significantly higher ($p<0.05$) in sorted semen supplemented with 0.75 mM GSH than that in the control. The percentages of moribund, dead and Phosphatidylserine (PS) translocated sperm detected by flow cytometry were significantly decreased ($p<0.05$) in frozen semen supplemented with GSH compared to the control. Higher blastocyst and pregnancy rates ($p<0.05$) were found after IVF and AI with frozen sperm treated with 0.75 mM GSH than that in the control group. In conclusion, addition of 0.75 mM GSH to the semen extenders (stained, sorted and frozen) during the sperm sorting process can improve sperm quality *in vitro* embryonic development and *in vivo* fertility after AI thus indicating potential for commercial application in buffalo sperm sorting.

Key words: Reduced glutathione, sperm quality, embryonic development, pregnancy rate, sorted semen, buffalo

INTRODUCTION

Millions of buffalo, mostly of the swamp type have been used as working animals in the traditional agriculture of Southern China (Lu *et al*., 2010). However, with the development of agricultural mechanization and urbanization, the physical performance of Chinese swamp buffalo is now insufficient to satisfy current commercial needs. Thus, it has become an urgent task of the current Chinese government to develop new genetic and reproductive technology to convert Chinese swamp buffalo to milk production. The establishment of buffalo sperm sorting technology (Lu *et al*., 2006, 2007, 2010; Liang *et al*., 2008) provides a good technical basis from which to accelerate this process of change. The application of sex control technology, combined with other reproductive biotechnologies such as IVF, AI, embryo transfer, etc. can double the rate of production of milk buffaloes thus resolving the shortage of milk buffaloes in China and promoting the development of the dairy industry in southern China. Although, the flow-cytometry technology for buffalo sperm sorting is well established, the blastocyst development rate after IVF and the conception rate after AI with sorted sperm are still lower than that with unsorted sperm (Lu *et al*., 1999, 2007; Dejarnette *et al*., 2011), suggesting that there may be damage to the sperm organelles during the sorting procedure.

Potential hazards in the procedure for flow-cytometric sorting of X and Y-sperm arise from high levels of dilution, nuclear staining, exposure to UV laser beams, high pressure and cryopreservation (Seidel and Garner, 2002; Spinaci *et al*., 2005). Data from earlier research has demonstrated that cryopreservation could cause overproduction of Reactive Oxygen Species (ROS) (Chatterjee and Gagnon, 2001) with oxidative damage to sperm resulting from an imbalance between ROS generation and scavenging (Roca *et al*., 2005). The main antioxidant enzymes involved in bovine semen are catalase, Glutathione Peroxidase (Gpx) and Superoxide Dismutase (SOD) (Bilodeau *et al*., 2000) and all of these antioxidants are highly diluted in the sheath fluid in sorted

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sperm (Klinc and Rath, 2007). In addition, antioxidant substances existed in the seminal plasma are lost because of the concentration of sperm after the sorting process by centrifugation. Thus, sperm cryopreservation results in decreased motility (Chatterjee and Gagnon, 2001) and plasma membrane integrity (Cerolini *et al*., 2001) and increased DNA fragmentation (Thomson *et al*., 2009) and sperm damage as a result of cryopreservation may arise because of excessive production of ROS (which is sensitive to oxidative damage because of its unsaturated fatty acid rich membrane (Jones *et al*., 1979; Chatterjee and Gagnon, 2001)) and a significant decrease in intracellular GSH content (Bilodeau *et al*., 2000).

Glutathione, a tripeptide thiol is present in all animal cells and plays an important role in the mediation of oxidative stress (Irvine, 1996). The present study reports an evaluation of the effects of GSH supplemention in stained, sorted and frozen semen extenders during the sperm sorting process on buffalo sperm quality (motility, plasma membrane integrity, apoptosis and DNA integrity), embryonic development after IVF and conception rate after AI.

MATERIALS AND METHODS

All chemicals used in this study were obtained from Sigma-Aldrich (USA) unless otherwise stated. All of the following experiments were replicated five times.

Semen preparation: Semen was collected from a fertility proven Nili-Ravi buffalo at the Livestock and Poultry Breeding Station of Guangxi, China. Sperm sorting was performed according to the report of Lu *et al*. (2006).

Semen staining: Briefly, semen was diluted with modified TALP (Tyrodes, Albumin, Lactate and Pyruvate) solution to a concentration of 200×10^6 spermatozoa/mL. The diluted semen was then stained with 40 μg mL^{-1} Hoechst 33342 with or without supplementation with GSH (0.75 mM reduced glutathione, Roche Diagnostics Corp), incubated at 34°C for 45 min and then further diluted in 2 mL of 4% egg yolk TALP containing 5% food dye (FD&C # 40). These semen samples were filtered through a 50 μm cell strainer to remove any agglutinated pieces of sperm.

Semen sorting: Flow sorting was performed using a flow cytometer (SX-MoFlo, Dako Cytomation, Inc., Fort Collins, CO, USA) equipped with an argon laser (ultraviolet 351 nm at 150 mW). Sorted sperm were collected in 50 mL tubes containing 2 mL of 20% TRIS-egg yolk extender supplemented with or without 0.75 mM GSH, chilled at 4°C for 1 h then centrifuged at 850×g at 4°C for 20 min.

Semen freezing: The centrifuged semen were resuspended in 20% TRIS-egg yolk extender containing 6% glycerol to a concentration of 8×10^6 spermatozoa/mL with or without supplementation with 0.75 mM GSH. Sperm were frozen in 0.25 mL-straws containing 2×10^6 spermatozoa and plunged into liquid nitrogen for storage.

Assessment of sperm quality: Sorted frozen sperm were thawed in a 37°C water bath for 30 sec then resuspended in DPBS to a final concentration of 1×10^7 spermatozoa/mL and held in a water bath at 27°C until evaluation. This was performed using a FACSCalibur flowing cytometer (Becton Dickinson, USA) and a fluorescence microscope (Nikon, Japan) to detect sperm plasma membrane integrity, apoptosis and DNA fragmentation.

Assessment of sperm motility by CASA: The proportions of sperm with total motility (MT, %) and progressive motility (MP, %) were assessed by computer-assisted semen analysis (CASA, Hamilton Thorne CEROS, USA) on 5 μL aliquots (10×10^6 spermatozoa/mL) placed on a pre-warmed (37°C) Leja four chamber slide (depth 20 μm).

Assessment of sperm plasma integrity: Plasma membrane integrity of buffalo sperm was assessed with SYBR-14 and Propidium Iodide (PI) using a LIVE/DEAD Sperm Viability kit (Molecular Probes, USA). Aliquots of 200 μL sperm sample (1×10^7 sperm/mL) were suspended in Hepes-buffer (10 mM Hepes, 150 mM NaCl, 10% BSA, pH 7.4) and stained with 1 μL SYBR-14 (100 nM) for 10 min at 37°C in the dark. After incubation, 1 μL of PI (12 μM) was added to the sperm suspension for 5 min. A minimum of 10,000 sperm in stained samples were assessed by flow cytometry to determine the percentages of viable (SYBR+), moribund (SYBR+/PI+) and dead spermatozoa (PI+).

Sperm apoptosis: Sperm were evaluated for apoptosis using an Annexin V-FITC/PI kit (BD Company, USA) following the protocol recommended by the manufacturer. Briefly, an aliquot (200 μL) of a sperm sample was centrifuged at 1500 rpm/min for 3 min. These sperm pellets were resuspended in 1×Annexin V binding buffer (1×10^7spermatozoa/mL) and 10 μL of Annexin-V solution. An aliquot of PI solution (10 μL) were added to 200 μL of the semen sample and after gentle mixing incubated for 15 min at room temperature. After that one aliquot of

binding buffer (800 μL) was added to the tube. A minimum of 10,000 sperm in stained samples were assessed by flow cytometry and classified as four different sperm subpopulations: live (AN-/PI-), live but PS translocated (AN+/PI-); dead and Phosphatidylserine (PS) translocated (AN+/PI+), dead and late necrotic (AN-/PI+).

Sperm DNA fragmentation: The Sperm Chromatin Dispersion test (SCD) was conducted according to the instructions of the SPERM-HALOMAX kit (Halotech, Spain). Briefly, 20 μL of sperm sample (10×10^6 spermatozoa/mL) was added to 10 μL of melted agarose (37°C). A drop of the sperm-agarose mixture was placed on the treated face of the slide cooled at 4°C and covered with a coverslip for 5 min at 4°C. The coverslip was then removed carefully and the slide set in 10 mL of the lysing solution for 20 min at room temperature (22°C) after which it was washed with distilled water for 5 min. Finally, the slide was dehydrated sequentially in 70, 90 and 100% ethanol for 2 min, air dried and stained in wright-giemsa and phosphate buffer (1:1, v/v) solution for 25 min. A minimum of 1,000 sperm were detected per sample under the x20 objective of the microscope. A large and spotty halo of chromatin dispersion was considered as a spermatozoa with fragmented DNA. A small and compact halo of chromatin dispersion was considered as spermatozoa with intact DNA.

Embryo production

***In vitro* maturation:** Buffalo oocytes were aspirated from follicles with 2-8 mm diameter using slaughterhouse derived ovaries and washed twice in TCM 199 (Gibco, Invitrogen Co., USA) supplemented with 5% Estrous Cow Serum (ECS). Cumulus Oocyte Complexes (COCs) were matured for 22-24 h in 100 μL of TCM-199 supplemented with 5% ECS, 15 μg mL^{-1} FSH and 1 μg mL^{-1} E_2 at 39°C, 5% CO_2. The COCs were removed from the drops following maturation and stripped off the cumulus cells.

Sperm preparation and insemination: Frozen semen with or without GSH were thawed at 37°C for 30 sec and treated by a swim-up procedure in modified Tyrode's medium supplemented with 0.6% BSA, 2.0 mM caffeine and 20 μg mL^{-1} heparin and incubated for 30 min at 39°C, 5% CO_2. After incubation, the supernatant was collected and centrifuged at 400× g for 5 min. The concentration of sperm added to the fertilization drop was 2×10^6/mL. About ten to fifteen oocytes were inseminated in 50 μL drops of modified Tyrode's medium for 8-10 h at 39°C, 5% CO_2.

***In vitro* culture:** Presumptive zygotes were co-cultured with buffalo granulosa cell monolayers in TCM-199 supplemented with 10% estrous cow serum at 39°C, 5% CO_2. Cleavage and blastocyst rates were examined at day 2 and 6-8 after insemination, respectively.

Deep uterine horn Artificial Insemination (AI): Buffaloes of spontaneous estrous were inseminated by the same skilled technician. For AI, 70 buffaloes were randomly distributed to two different groups. Low dose frozen sperm (2×10^6 spermatozoa) with or without GSH was deposited into the uterine horn ipsilateral to the side of dominant follicle by transrectal palpation. AI was performed twice with an interval of 12 h for each estrus and the first insemination was done 8 h after overt signs of heat. Diagnosis of pregnancy was determined at 90 days after AI by transrectal palpation.

Statistical analysis: Statistical analyses were conducted using SPSS 17.0 for Windows (Statistical Analysis Software package, USA). Treatment effects were assessed by one-way analysis of variance except for conception rate with the χ^2-test. Statistical significance was set at $p = 0.05$ and values are presented as the average±standard deviation.

RESULTS AND DISCUSSION

The effect of GSH supplementation on progressively motile sperm in stained, sorted and frozen semen extenders is shown in Table 1. The percentage of sperm with progressive motility was significantly higher ($p<0.05$) in sorted semen supplemented with GSH than that in the control. GSH supplementation of semen extenders had no significant effect on plasma membrane integrity (stained, sorted and frozen) in measurements by flow cytometry ($p>0.05$) (Fig. 1). However, the percentage of moribund sperm (SYBR+/PI+) was significantly decreased ($p<0.05$) when supplemented with GSH in frozen semen compared to that in the control (Fig. 1). Also, in frozen semen supplemented with GSH, the percentage of live spermatozoa (A-/PI-) was significantly higher ($p<0.05$) and that in late apoptosis (A+/PI+) significantly lower ($p<0.05$) (Fig. 2). GSH supplementation had no significant effect on

Table 1: Effects of GSH supplementation during semen sorting on progressive motility and DNA fragmentation in buffalo sperm for stained, sorted and frozen semen extenders (mean %±D)

Treatment groups	MP (%)	DNA fragment (%) (SCD)
Stained (control)	86.94±1.98	2.25±1.67
Stained (GSH)	86.85±4.69	1.94±0.19
Sorted (control)	39.07±3.00[a]	0.47±0.53
Sorted (GSH)	50.76±3.23[b]	0.46±1.38
Frozen (control)	31.10±2.25	2.26±3.70
Frozen (GSH)	38.29±3.49	1.42±1.78

MT: Total Motility; MP: Progressive Motility. Different letters (a-b) indicate significant difference within the groups ($p<0.05$)

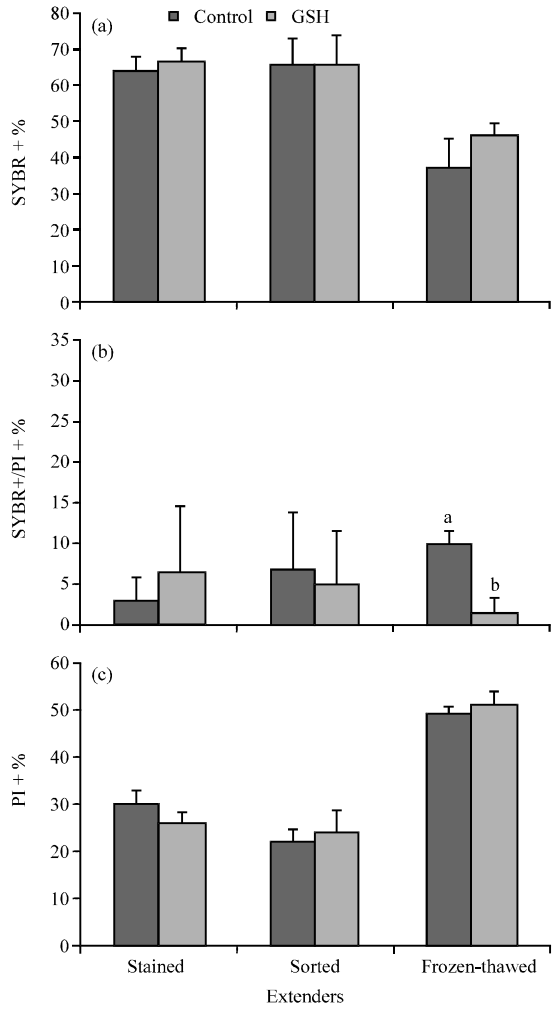

Fig. 1: Effects of GSH supplementation during semen sorting on buffalo sperm membrane integrity in stained, sorted and frozen extenders by flow cytometry (mean %±SD); a) SYBR + %; b) SYBR+/PI + %; c) PI + %. Different letters (a-b) indicate significant difference within the groups ($p<0.05$)

the numbers of sperm with DNA fragmentation ($p>0.05$) in the semen extenders (stained, sorted and frozen) (Table 1 and Fig. 3).

There were no significant differences ($p>0.05$) in cleavage rate between the control and the sperm treated with GSH in the stained, sorted and frozen semen extenders after IVF (Table 2) but the blastocyst rate was increased ($p<0.05$) in the sperm treated with GSH (Table 2). A significantly higher conception rate ($p<0.05$) was found in X sperm treated with GSH than that in controls but there was no significant difference in the abortion rate ($p>0.05$) (Table 3).

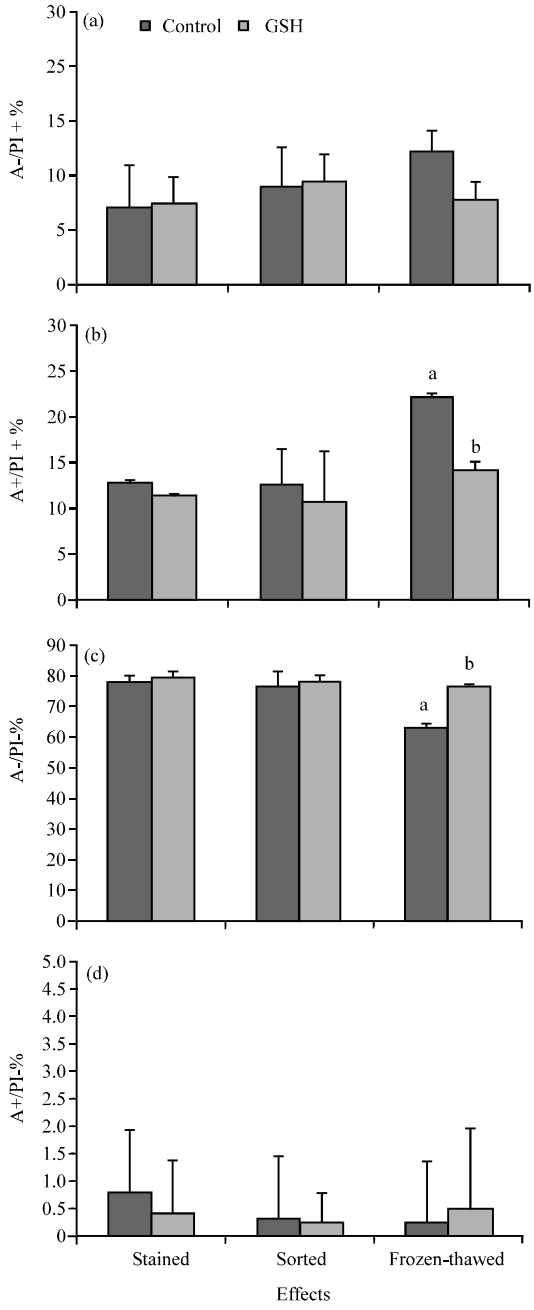

Fig. 2: Effects of GSH supplementation during semen sorting on apoptosis of buffalo sperm in stained, sorted and frozen extenders by flow cytometry (mean %±SD). a) A-/PI+%; b) A+/PI+%; c) A-/PI-% and d) A+/PI-%. Different letters (a-b) indicate significant difference ($p<0.05$)

Low viability in sorted compared to unsorted sperm is one of mainly factors caused by Reactive Oxygen Species (ROS) which have been shown to increase as

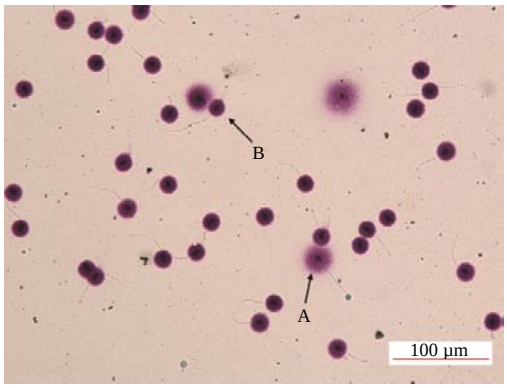

Fig. 3: Effects of GSH supplementation during semen sorting on DNA fragmentation of buffalo sperm in stained, sorted and frozen extenders by Sperm Chromatin Dispersion test (SCD). A) sperm with DNA fragmentation and B) sperm with intact DNA

Table 2: Effects of GSH supplementation during semen sorting on the number of oocytes, cleavage and blastocyst rates after IVF in buffalo (mean %±SD)

Treatment groups	No. of oocytes	Cleavage (%)	Blastocysts/oocyte (%)
Control	192	43.6±8.0	9.74±1.86[a]
GSH	180	40.5±2.4	13.67±1.95[b]

Table 3: Effects of GSH supplementation during semen sorting on pregnancy and abortion rates in buffalo (mean %±SD)

Treatment groups	No. of buffaloes	No. of pregnant (%)	No. of abortion (%)
Control	35	10 (28.6)[a]	1 (2.9)
GSH	35	20 (57.1)[b]	1 (2.9)

Different letters (a-b) indicate significant difference ($p<0.05$)

a result of centrifugation and cryopreservation (Agarwal *et al*., 1994; Chatterjee and Gagnon, 2001). Also, a reduction in oxidative stress has been observed in bovine sperm in the present of antioxidant substances during the flow sorting procedure (Klinc *et al*., 2007; Klinc and Rath, 2007). Furthermore, cryopreservation results in reductions in the levels of glutathione in bovine (Bilodeau *et al*., 2000; Stradaioli *et al*., 2007) and boar sperm (Gadea *et al*., 2004). Therefore, the objective of this study was to determine the extent to which supplementation with GSH in stained, sorted and frozen semen extenders could improve sperm quality.

The present results indicate that the percentage of progressive motility was significantly higher in sorted semen supplemented with GSH than in the control. Previous research reported that GSH supplementation in TALP extender improved progressive motility of bull sperm during storage at 25°C (Foote *et al*., 2002).

The data from the present study using flow cytometry showed that in the frozen semen extender supplemented with GSH, the percentage of live spermatozoa (A-/PI-) was significantly higher than that in the control whilst the percentage of sperm in late apoptotic state (A+/PI+) was lower ($p<0.05$). Supplementation of the dilution medium with GSH has been reported to reduce ROS generation in frozen-thawed bull (Gadea *et al*., 2008) and boar (Gadea *et al*., 2005) sperm. Apoptosis has been correlated positively with ROS in human sperm (Moustafa *et al*., 2004) and this may be the explanation for the reduced level of apoptosis in frozen semen supplemented with GSH in the present study.

The present results show GSH supplementation in the semen extenders (stained, sorted and frozen) had no significant on the number of sperm with DNA fragments. Previously Boe-Hansen *et al*. (2005) reported that the sorted X and Y sperm had a significantly lower number of DNA fragments compared with the conventional bovine semen but is has also been reported that bovine sperm possess a highly compact and stable chromatin (Celeghini *et al*., 2008).

There is some evidence that GSH supplementation in the semen extenders (stained, sorted and frozen) could support the development of embryos *in vitro*. It had earlier been showed that addition of 5 mM GSH to the thawing extender improved morulae and blastocysts development compared to the control (Gadea *et al*., 2008) and supplementation with 1 mM GSH in IVF medium (Kim *et al*., 1999) or culture media (Luvoni *et al*., 1996) could increase the blastocyst development *in vitro*. The results showed that supplementation with 0.75 mM GSH in the stained, sorted and frozen semen extenders resulted in an increase of blastocyst formation after IVF.

Even when using high-speed flow cytometry only 4500-5500 spermatozoa/sec would have been separated with purity of X and Y at 90% and the total of 1×10^7 of sorted spermatozoa took >30 min. Therefore, sperm were exposed to aerobic conditions for a long time during the flow sorting procedure and this is the likely reason for potential damage by ROS. Such damage does not occur during natural mating when sperm are under anaerobic conditions. In addition, high concentrations of taurine have been found in oviduct fluids and this also plays an important role in protecting sperm from attack by ROS in aerobic conditions (Holmes *et al*., 1992). Conception rate was improved after AI with X sperm treated with GSH in the stained, sorted and frozen semen extenders thus supporting the concept that GSH scavenges ROS produced as a result of exposure to aerobic conditions during the flow sorting procedure. The results are also consistent with earlier reports which showed that apoptotic sperm was negatively related to fertility (Anzar *et al*., 2002).

CONCLUSION

The results of this study show that the percentage of sperm with progressive motility is much higher in sorted semen supplemented with GSH than in the control. Also, the percentage of apoptosis was decreased supplemented with GSH in frozen semen and the blastocyst and pregnancy rates were improved when the sperm was treated with GSH in the stained, sorted and frozen semen extenders after IVF and AI.

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