

Technology Report

Effects of glutathione treatments during sperm washing and *in vitro* fertilization on the *in vitro* early development of embryos of Japanese Black cattle

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Abstract. This study was conducted to examine the effects of adding glutathione (1 mM) to media used for sperm washing and *in vitro* fertilization (IVF) on the improvement of early development of embryos produced using cryopreserved spermatozoa of the less IVF-competent bull (the one considered unqualified as spermatozoa supplier for the production of bovine blastocysts using IVF). The cryopreserved spermatozoa of this bull were characterized by normal motility and lower ATP content and blastocyst productivity than those of IVF-competent bulls. The addition of glutathione to the sperm washing medium was more effective in improving the productivity of blastocysts and ATP content than the addition of glutathione to the IVF medium or no glutathione addition at all (control). These results suggest that this simple method may be used to improve the potential of cryopreserved spermatozoa of less IVF-competent bulls to fertilize oocytes *in vitro* and to induce normal embryonic development after fertilization.

Key words: Adenosine triphosphate concentration, Glutathione treatment, *In vitro* fertilization, Japanese Black bull, Sperm washing

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In cattle reproduction, ejaculated spermatozoa of sires are routinely cryopreserved and later used for artificial insemination. However, excessive reactive oxygen species (ROS) are produced during freezing and thawing; they are considered as one of the most deleterious factors of cryopreservation for human [1] and bovine [2] spermatozoa, leading to decreased sperm motility and intracellular reduced glutathione (GSH) content. A study showed that the GSH content of bovine spermatozoa decreased by approximately 20% during cryopreservation [3]. Therefore, exogenous GSH is routinely added to the semen extender to maintain sperm motility after thawing [4]. Moreover, ROS have deleterious effects on other sperm aspects, including plasma membranes (causing lipid peroxidation) [5], paternal genomes (inducing DNA denaturation) [6], the ability to fertilize oocytes [7], and the potential to induce normal embryonic development after fertilization [8].

A low molecular-weight scavenger such as GSH protects spermatozoa, oocytes, and embryos against ROS that cause oxyradical damage. Furthermore, GSH promotes the decondensation of sperm chromatin in fertilized eggs by the reduction of disulfide bonds. When ROS levels are increased in the spermatozoa, chain reactions of oxidative stress are often investigated, including lipid peroxidation and damage to plasma membranes, leakage of intracellular enzymes, and chromatin damage [5]. Their effects are linked to the depletion of intracellular adenosine triphosphate (ATP) and, consequently, to an irreversible loss of sperm motility [6] and a reduction in sperm fertilizing ability [7].

For bovine *in vitro* fertilization (IVF), the blastocyst development rate is used as an index of successful bovine IVF. Several investigations have shown that sperm motility affects the results of IVF and early embryonic development [8]. The ability of cryopreserved spermatozoa to fertilize oocytes *in vitro* and to induce normal early development of fertilized eggs to the blastocyst stage have been shown to be largely different among sperm supplies (bulls) [9]. Therefore, to improve blastocyst development rates, it is necessary to develop a new method to improve poor sperm samples with deficient abilities.

It has been reported that, when GSH is used to treat spermatozoa, the antioxidant capacity of bull semen increases, leading to significant increases in the number of 4- to 8-cell stage embryos formed and in blastocyst development rate, and decreases in the ratio of DNA fragmentation [9]. Luvoni *et al.* [10] showed a positive effect on blastocyst development when GSH was added to *in vitro* embryo cultures, but not when added during *in vitro* maturation (IVM) or IVF. Others reported an increase in blastocyst formation when GSH was present in the sperm preparation medium [11] or during IVF procedures [12]. Kim *et al.* reported that the effect of GSH (1 mM) during IVF on embryonic development was dependent on sperm suppliers [13]. However, the cause of variation on the effect of GSH remains unclear. Therefore, it is necessary to perform additional studies on sperm treatment in bovine IVF. In the present study, we examined the effect of treatment with GSH during bovine IVF on embryonic development of eggs fertilized with spermatozoa from a less IVF-competent bull.

In Experiment 1, we compared the results of IVF and embryo production using slaughterhouse-obtained oocytes. The blastocyst development rate after IVF was examined for 10 bulls; the blastocyst development rate for bull J was significantly lower than that for bulls A–E (14.5 vs. 41.2–30.9, $P < 0.05$; Fig. 1A). The ATP concentrations of spermatozoa from the 10 bulls were measured. The ATP concentrations in bulls A and B were significantly higher than those in other bull spermatozoa samples, whereas the sample

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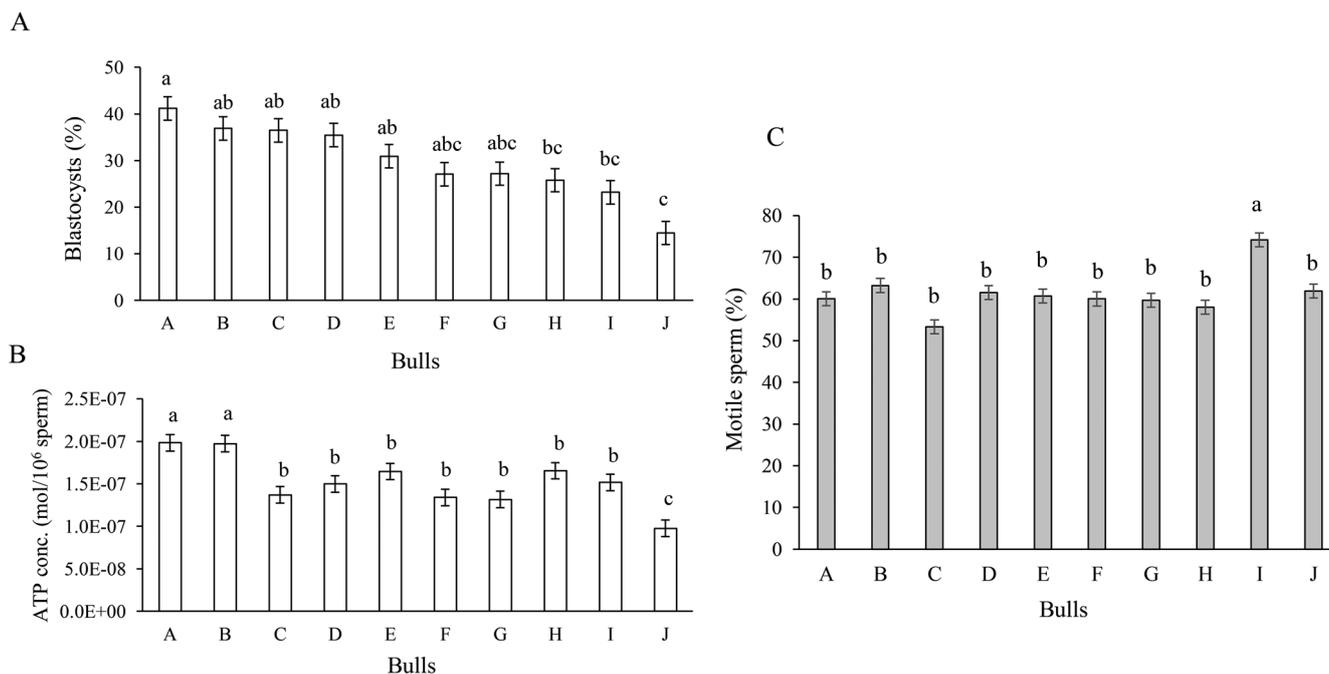


Fig. 1. Variations in the characteristics of frozen-thawed spermatozoa samples from 10 different bulls. (A) Blastocyst formation rates among oocytes fertilized *in vitro* with frozen-thawed spermatozoa. (B) Adenosine triphosphate (ATP) concentration in frozen-thawed spermatozoa washed twice with mTALP medium containing caffeine. ATP concentrations were measured by a luciferase bioluminescence assay. (C) Motility of frozen-thawed spermatozoa from 10 different bulls. Motility was evaluated using a computer-aided sperm analyzer. Bars represent the mean \pm SEM (a–c: different lowercase letters above bars indicate statistically significant differences, $P < 0.05$). Statistical analyses were done using one-way ANOVA and the Tukey's multiple comparison test, $n = 3$.

from bull J had a significantly lower concentration (0.9760×10^{-7} mol/10⁶ sperm vs. 1.9839×10^{-7} – 1.3168×10^{-7} mol/10⁶ sperm, $P < 0.05$; Fig. 1B). Furthermore, a possible relationship between ATP and sperm concentrations was investigated among the 10 bulls, apparently indicating no correlation between these parameters. In addition, sperm motility assays on frozen-thawed spermatozoa of 10 bulls using a computer-aided sperm analyzer (CASA) system showed that the percentages of motile spermatozoa did not vary significantly (Fig. 1C). As shown in Figs. 1A and 1B, our results showed that there was a tendency for spermatozoa with higher ATP concentrations to have higher cleavage and blastocyst development rates. Interestingly, Sonderquist and Stalhammar showed that there was a significant correlation between ATP concentration and motility in bovine spermatozoa [14]. ROS may cause lipid peroxidation of the sperm plasma membrane (affecting sperm motility and penetration and fusion with oocytes) and even DNA damage [7]. However, in our experiments, using frozen-thawed spermatozoa of Bull J, treatments with GSH scarcely affected sperm motility (data not shown), whereas treatments significantly affected ATP concentration in sperm, DNA denaturation (evaluated through acridine orange (AO) staining), and blastocyst development rates (see the results of Experiment 2).

In Experiment 2, we examined the effect of GSH addition on embryonic development using spermatozoa from Bull J, which had a low embryo development performance. A conventional IVF method was used in the control group without GSH. This experiment was designed to examine the effects of adding 1 mM GSH to the medium used for sperm washing (*i.e.*, the first step in sperm processing; sperm washing medium: mTALP medium containing 10 mM caffeine), to the medium used for IVF procedures (IVF medium: mTALP medium containing heparin and caffeine), or to both media on early development of IVF embryos. Table 1 shows the results of

embryonic development after IVF. The cleavage rates were 82.4% for the sperm washing medium group (supplemented with 1 mM GSH), 73.4% for the IVF medium group (supplemented with 1 mM GSH), 72.1% for the sperm washing medium + IVF medium group (both media supplemented with 1 mM GSH), and 47.4% for the control medium group. Moreover, the blastocyst development rates were 39.5%, 24.8%, 27.3%, and 15.8% in the sperm washing medium, IVF medium, sperm washing medium + IVF medium, and control medium groups, respectively. The results obtained for all the experimental groups regarding cleavage and blastocyst development rates were significantly higher than those for the control group. In addition, the total number of cells in the blastocysts was significantly higher in the sperm washing medium group than in the control medium group (Table 1). These results suggest that the cleavage and blastocyst development rates are improved by GSH media supplementation. Moreover, GSH-supplemented sperm washing medium may improve embryonic development and produce good-quality IVF embryos.

GSH not only reduces ROS, but also disulfide bonds [15]. Apparently, the promoting effects of GSH on embryonic development are dependent mainly on the effective reduction of disulfide bonds in spermatozoa. Therefore, the function of GSH is important for sperm chromatin decondensation following sperm penetration of the oocyte, destabilization and replacement of protamines by oocyte-derived histones, and the development of the sperm nucleus into the male pronucleus [16]. Our findings indicate that the potential of frozen-thawed spermatozoa from less IVF-competent bulls to induce normal embryonic development can be improved simply by adding GSH to the sperm washing medium (treatment time: 5 min).

In Experiment 3, we examined the effects of the addition of GSH to the sperm washing medium on the ATP concentration of spermatozoa. Figure 2A shows that the ATP concentration of spermatozoa treated

Table 1. Effects of adding GSH to the media used for sperm washing and for *in vitro* fertilization (IVF) on the *in vitro* early development of IVF embryos and blastocyst cell number

Media supplemented with GSH	No. of oocytes	No. (%) of cleavages	No. (%) of blastocysts	No. of blastocysts used for cell counting	No. of cells		
					TE	ICM	Total
Sperm washing	119	98 (82.4 ± 3.7) ^a	47 (39.5 ± 2.3) ^a	36	105.1 ± 4.1	52.0 ± 3.0	157.0 ± 3.8 ^a
IVF	109	80 (73.4 ± 6.1) ^a	27 (24.8 ± 3.4) ^b	14	105.5 ± 9.0	38.7 ± 5.5	144.2 ± 10.2 ^{ab}
Sperm washing + IVF	154	111 (72.1 ± 4.9) ^a	42 (27.3 ± 2.8) ^{ab}	13	93.7 ± 4.4	47.4 ± 3.5	141.1 ± 3.4 ^{ab}
Control	114	54 (47.4 ± 4.0) ^b	18 (15.8 ± 1.3) ^c	13	94.9 ± 9.4	37.9 ± 3.8	132.8 ± 10.0 ^b

The experiments were performed using spermatozoa from bull J. The cleavage rates, blastocyst development rates, and blastocyst cell numbers are shown as the mean ± SEM. Different superscript letters in the same column (a, b, or c) indicate statistically significant differences ($P < 0.05$), $n = 3$. Cleavage and blastocyst formation rates were evaluated 72 and 192 h after IVF, respectively. TE, trophectoderm; ICM, inner cell mass.

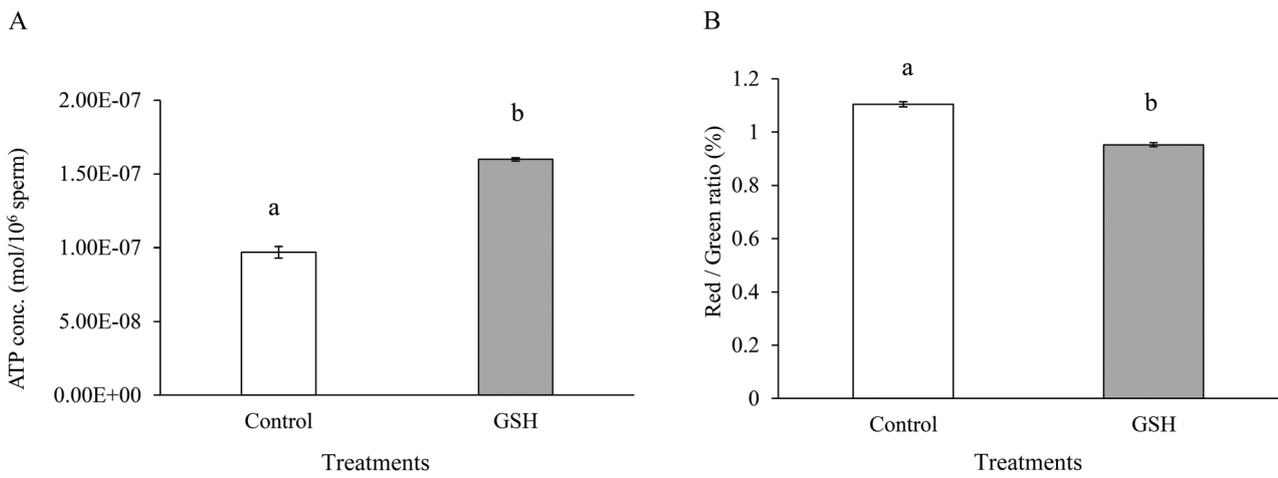


Fig. 2. Effect of glutathione (GSH)-supplemented sperm washing medium on ATP concentration and DNA denaturation degree in frozen-thawed spermatozoa from bull J. (A) ATP concentration of spermatozoa after being washed twice with a medium with or without GSH. (B) AO nuclear staining (for determining DNA denaturation degree) of spermatozoa after being washed with a medium with or without GSH. At least 100 sperm nuclei were analyzed in each experimental group by fluorescence microscopy. Bars represent the mean ± SEM (a, b: different lowercase letters above bars indicate statistically significant differences, $P < 0.05$). Statistical analyses were done using the Student's *t*-test, $n = 3$.

with 1 mM GSH for 5 min ($1.5984 \pm 0.0125 \times 10^{-7}$ mol/ 10^6 sperm) was significantly higher than that of control spermatozoa ($0.9699 \pm 0.0384 \times 10^{-7}$ mol/ 10^6 sperm). Soderquist and Stalhammar [14] reported a significant correlation between ATP concentration and motility in bovine spermatozoa. Moreover, Foresta *et al.* showed that sperm incubation with extracellular ATP induced a significant increase in fertilization rates when following IVF techniques, further supporting the important role of this nucleotide in sperm activation and oocyte fertilization [17]. Therefore, through the addition of GSH to spermatozoa for only 5 min, the ATP content was increased and, after IVF of oocytes, embryonic development performance was improved.

For AO staining, smears were prepared after sperm washing, and staining was performed after DNA denaturation with hydrochloric acid. Thereafter, the red and green luminances of the sperm heads were measured, and the red to green ratio was calculated. The higher the ratio, the greater the DNA denaturation degree. The sperm washing group had significantly less denatured DNA than the control group (Fig. 2B). Given that AO staining reflects the thiol-disulfide status of sperm nuclei [18, 19], the results of this experiment suggest that GSH treatment may have had protective effects on sperm head DNA. Furthermore, as spermatozoa are particularly susceptible to oxidative stress (that leads to chromatin damage), GSH may be able to protect sperm chromatin before penetration.

In conclusion, the addition of GSH to the sperm washing and IVF media significantly improved the function and fertilization ability of bovine frozen-thawed spermatozoa. In the future, we hope to develop a novel treatment for sperm to eliminate variations in embryonic development after IVF using frozen-thawed spermatozoa from different sperm suppliers.

Methods

IVM, IVF, and embryo culture

Bovine ovaries from Japanese Black cows were obtained from a local slaughterhouse. Cumulus oocyte complexes (COCs) were matured *in vitro*, following a previously described method [15]. Frozen-thawed spermatozoa of Japanese Black bulls were washed twice with mTALP medium supplemented with 10 mM caffeine (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) by centrifugation at $500 \times g$ for 5 min. The resultant sperm pellet was resuspended in the same medium at a final concentration of 20×10^6 sperm/ml. An equal volume of mTALP medium supplemented with 3 mg/ml bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) and 10 IU/ml heparin (NOVO Heparin, Mochida Seiyaku, Tokyo, Japan) was added to the sperm suspension. Groups of COCs matured *in vitro* were introduced into 100- μ l microdrops of sperm suspension in a 35 mm culture dish at 38.5°C in a 5%

CO₂ atmosphere [15]. Six hours after the initiation of insemination, cumulus cells were removed from the oocytes using a pipette.

The culture medium was composed of modified synthetic oviduct fluid (mSOF) [20, 21] supplemented with 20 µl/ml essential amino acid solution (50 ×, Thermo Fisher Scientific, Waltham, MA, USA), 10 µl/ml nonessential amino acid solution (100 ×, Thermo Fisher Scientific), 1 mM glycine, 2 mM taurine, insulin-transferrin-selenium supplement (final concentrations of 5 ng/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium; Sigma-Aldrich), and 6 mg/ml fatty acid-free BSA (Sigma-Aldrich). Oocytes were cultured in groups of 10–15 in 50 µl drops of mSOF medium at 38.5°C in a 5% CO₂, 5% O₂, and 90% N₂ atmosphere.

Differential staining of inner cell mass (ICM) and trophectoderm (TE) cells

Blastocysts were stained for 30 sec with 100 µg/ml propidium iodide (Sigma-Aldrich) in a permeabilizing 0.2% (v/v) Triton X-100 (Sigma-Aldrich) solution. Blastocysts were then counterstained for 5 min with 25 µg/ml Hoechst 33342 (Calbiochem, La Jolla, CA, USA) and simultaneously fixed in 99.5% ethanol. After blastocyst mounting, ICM and TE cells were counted by fluorescence microscopy [22].

ATP assays

The ATP content of spermatozoa was measured using an ATP-dependent luciferin-luciferase bioluminescence assay (ATP Bioluminescent Assay kit; Sigma-Aldrich). For each sample, including the control and all GSH treatment groups, 10 × 10⁶ spermatozoa were prepared; 0.1 ml ATP Assay Mix Solution was added to each vial. Determinations were done in duplicates. The mixture was incubated at room temperature (25–30°C) for 3 min and mixed; sample luminescence was measured immediately using a luminometer (Gene Light 55; Microtech Co., Ltd., Chiba, Japan). Sample mean values were calculated using data from the two vials from the same sperm sample as previously described [15].

AO staining

Spermatozoa were gently smeared on glass slides, air-dried, and fixed overnight in freshly prepared Carnoy's solution (methanol: glacial acetic acid, 3:1). AO (Sigma-Aldrich) staining solution (final concentration, 0.19 mg/ml) was freshly prepared by adding 10 ml of stock solution (1 mg/ml AO in distilled water) to 40 ml of 0.1 M citric acid + 2.5 ml of 0.3 M Na₂HPO₄·7H₂O and adjusting the pH to 2.5 [23]. The smeared spermatozoa were treated with AO solution in the dark for 10 min at room temperature and then gently rinsed with deionized water. The slides were sealed with coverslips and observed under a fluorescence microscope (Nikon, Tokyo, Japan). The areas covered by red and green fluorescence signals within each nucleus were measured using ImageJ (NIH, Bethesda, MD, USA) and expressed as the relative fluorescence intensity. At least 100 sperm nuclei were evaluated under each experimental condition.

Sperm motility assay

Sample aliquots (4 µl each) were placed onto 4-chamber slides (Leja, Nieuw-Vennep, Netherlands). At least 200 sperm cells across five fields per chamber were counted for motile and dead sperm, and the percentage of motile sperm was determined using a CASA (Hamilton-Thorne Inc., Beverly, MA, USA) [14].

Statistical analyses

Data on the *in vitro* development rates (Table 1), differential staining of ICM and TE cells (Table 1), blastocyst development rates (Fig. 1A),

ATP concentrations (Fig. 1B), and sperm motility (Fig. 1C) of sperm samples from 10 different bulls were analyzed using one-way analysis of variance (ANOVA). Means were compared using the Tukey's multiple comparison test. Student's *t*-tests were used to analyze sperm ATP concentration (Fig. 2A) and DNA denaturation (Fig. 2B). All percentage data were arcsine-transformed prior to the ANOVA or Student's *t*-test. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Japan), which is a graphical user interface for a modified version of R Commander (version 1.6.3; The R Foundation for Statistical Computing) that includes statistical functions frequently used in biostatistics. Data were considered significantly different when P-values < 0.05.

Experimental design

Experiment 1: Frozen-thawed spermatozoa from 10 bulls were used in subsequent experiments. Blastocyst development rates after IVF were investigated using slaughterhouse-obtained oocytes. ATP concentrations in washed spermatozoa were examined. After freeze-thawing, sperm motility was measured using a CASA.

Experiment 2: Frozen-thawed spermatozoa from bull J were used in Experiment 2 using a conventional IVF method [24]. This experiment was designed to examine the effects of 1 mM GSH on the medium used for sperm washing (sperm washing medium: mTALP medium containing 10 mM caffeine), the medium for the IVF procedure (IVF medium containing heparin and caffeine), or both media on early development of IVF embryos (cleavage rates and blastocyst development rates at 72 h and 192 h after sperm insemination).

Experiment 3: Frozen-thawed spermatozoa from bull J were washed and centrifuged (at 500 × *g* for 5 min); first, with mTALP medium supplemented with 10 mM caffeine and 1 mM GSH and, second, with mTALP medium supplemented with 10 mM caffeine (without GSH). After washing, the spermatozoa were used to measure ATP concentrations and for AO staining.

Conflict of interests: The authors have no conflicts of interest to declare.

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