

Glutathione treatment of Japanese Black bull sperm prior to intracytoplasmic sperm injection promotes embryo development

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Abstract. Intracytoplasmic sperm injection (ICSI) was expected to enable more efficient use of sperm from sires with preferable genetic traits and result in a generation containing a larger number of offspring with superior genetic characteristics in livestock. However, the efficiency of the early development of embryos produced by ICSI is still far from satisfactory in cattle. The present study aimed to investigate the effects of the treatment of cryopreserved sperm with glutathione (GSH) on the early development of embryos produced by ICSI in Japanese Black cattle. Moreover, the disulfide bond state and mitochondrial function were investigated in the sperm treated with GSH to confirm the effectiveness of the abovementioned treatment. We also investigated the effect of 7% ethanol activation treatment on the developmental ability of ICSI embryos using GSH-treated sperm. There was no effect on the blastocyst rate from the activation treatment. When sperm-injected oocytes were cultured *in vitro*, the treatment with GSH significantly improved the early development of embryos. Specifically, the rates of embryos reaching the 4–8-cell stage and blastocyst stage were significantly higher in ICSI with GSH-treated sperm (71.4% and 31.0%, respectively) than that with the control sperm (36.6% and 7.0%, respectively). Moreover, the GSH-treated sperm treatment significantly decreased the number of disulfide bonds in the sperm head (as shown by monobromobimane staining) and enhanced the mitochondrial function in the sperm middle piece (as shown by Rhodamine 123 staining and the adenosine triphosphate-dependent bioluminescence assay). Based on these results, we suggest that the treatment of cryopreserved sperm with GSH might contribute to the improvement of ICSI techniques for the production of blastocysts in Japanese Black cattle.

Key words: Adenosine triphosphate concentration, Glutathione treatment, Intracytoplasmic sperm injection, Japanese Black cattle

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Intracytoplasmic sperm injection (ICSI) is a micro-injection technique that involves the direct injection of a spermatozoon into the ooplasm conducted under a microscope. To improve the efficiency of production of bovine embryos, previous studies have occurred on the methodology for embryo production using ICSI. One advantage of ICSI is the effective utilization of sperm from excellent sires, which can lead to an increase in the number of offspring of superior Japanese Black bulls. The benefit of the ICSI technique lies in the use of genetically and economically valuable sperm. However, the efficiency of the early development of embryos produced by this method is still far from satisfactory in cattle.

In contrast to conventional *in vitro* fertilization (IVF), ICSI involves the injection of a spermatozoon directly into the ooplasm without the occurrence of the acrosome reaction. The nucleus disulfide bridges in the sperm head after ICSI become reduced by glutathione (GSH) in the ooplasm [1, 2]. Consequently, the sperm head nuclei show

significant decrease in the disulfide bonds in the protamines, which are replaced by histones [3]. Subsequently, the male pronucleus is formed from the sperm head and becomes fused with the female pronucleus. Compared to the sperm of several other species (e.g., mouse, human, and hamster), bull sperm is more stable and does not readily decondense in bovine oocytes [1].

Various chemicals such as heparin [4–6], caffeine [4–8], and calcium ionophore [4, 6] increase sperm membrane permeability, acrosome reaction, and sperm head decondensation; these reagents are therefore routinely used in bovine IVF. In addition, the compound dithiothreitol (DTT) has been shown to induce the reduction of protamine disulfides in the sperm head [9–11]. Pretreatment of spermatozoa with DTT might therefore aid embryo development in sperm-injected oocytes [4, 12–14]. However, DTT is known to be a very strong reducing agent and embryos produced by ICSI from DTT-treated sperm are reported to have chromosomal abnormalities [12].

GSH is another common reducing agent and is a powerful antioxidant that counteracts the effects of oxidative stress [13, 14]. Oxidative stress results from increasing levels of reactive oxygen species, which lead to lipid peroxidation, intracellular enzyme leakage and, ultimately, chromatin damage [13, 14].

The majority of collected bovine sperm is cryopreserved and used for artificial insemination. However, during freezing and thawing,

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excessive reactive oxygen species are produced, which is considered one of the main factors contributing to the deleterious effect of low temperatures on human [15, 16] and bovine [17] sperm. Sperm motility and concentration of native GSH in sperm decreases with freeze–thawing cycles, with reported decreases in GSH content in bull sperm of up to 78% [17, 18]. GSH is therefore routinely added to the sperm prior to freezing to maintain motility after thawing [19].

For bovine IVF, it has also been reported that when GSH is used to treat sperm, the antioxidant capacity of bull semen increases, leading to significant increases in the 4–8-cell embryo development rate and blastocyst development rate, and decreases the ratio of DNA fragmentation [20].

The purpose of the present study was to investigate the effects of treatment with GSH on the embryo developmental ability after ICSI, the disulfide bond state, and mitochondrial function in cryopreserved sperm of Japanese Black cattle.

Materials and Methods

Collection of cumulus oocyte complexes (COCs)

Oocytes were matured *in vitro*, following methodology previously described [21, 22]. Bovine ovaries from Japanese Black cows or heifers were obtained from a local slaughterhouse and transported to the laboratory within 2 h. Cumulus oocyte complexes (COCs) were aspirated from antral follicles 2–8 mm in diameter through a 21-gauge, 1.58 cm (5/8 inches) needle attached to a 10 ml syringe.

Oocyte maturation

The isolated COCs were washed three times. Groups of 50 COCs were matured in 500 μ l TCM199 (Gibco BRL, Grand Island, NY, USA) supplemented with 4 mg/ml of bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), 0.1 IU/ml of follicle stimulating hormone (antrin; Kyoritsu Seiyaku, Tokyo, Japan), and 50 ng/ml of epidermal growth factor (Upstate Biotechnology, Lake Placid, NY, USA) in 4-well multi plates (Nunc, Roskilde, Denmark) under mineral oil (Nacalai Tesque, Kyoto, Japan) at 38.5°C in a 5% CO₂ atmosphere for 22 h.

Sperm preparation

Cryopreserved sperm straws from a Japanese Black bull were thawed at 37°C for 30 sec in a water bath. The sperm were washed with BSA-free modified Tyrode's albumin lactate pyruvate (mTALP) [23], supplemented with 10 mM caffeine (Wako Pure Chemical Industries, Osaka, Japan), and centrifuged at 500 \times g for 5 min [24]. Washed spermatozoa were then incubated with BSA-free mTALP supplemented with 8 mM GSH (Wako Pure Chemical Industries) for 10 min at 38.5°C [12, 25, 26] and washed again. GSH was not added to the control group. Pelleted sperm were resuspended in BSA-free mTALP supplemented with 10 mM caffeine. To prepare sperm for ICSI, 10 μ l of the sperm suspension was mixed with 30 μ l of 12% polyvinylpyrrolidone K90 (MP Biochemical, OH, USA).

ICSI

After oocyte maturation, cumulus cells were thoroughly dispersed with 0.1% bovine testicular hyaluronidase (Sigma-Aldrich) and removed from oocytes by gentle pipetting. After the removal of

cumulus cells, only oocytes with a visible first polar body were selected, and these were washed three times in TCM199 supplemented with 5% calf serum (CS; Gibco BRL) and stored in the same medium until further treatment at 38.5°C in a 5% CO₂ atmosphere.

During ICSI, oocytes were suspended in M2 medium supplemented with 5% CS (Gibco BRL). ICSI was performed using a piezo-driven micromanipulator (PMM-150; Prime Tech, Ibaraki, Japan) based on published methods [21, 22]. Immediately before sperm injection, a motile spermatozoon was immobilized by breaking its tail with the tip of the injection needle. An oocyte was secured using a pipette and the polar body was vertically positioned. The immobilized spermatozoon was then transported tail-first into the injection pipette. The zona pellucida was penetrated by applying several piezo pulses. The spermatozoon was pushed forward until its head approached the center of the oocyte. The oolemma was punctured using a single piezo pulse and the spermatozoon was injected into the ooplasm. Injected oocytes were transferred into 50 μ l TCM199 supplemented with 5% CS under paraffin oil and stored at 38.5°C in a 5% CO₂ atmosphere. We selected second polar body oocytes at 4 h following ICSI and excluded first polar body oocyte [21, 22].

Treatment for oocyte activation

The sperm-injected oocytes were cultured in TCM199 supplemented with 5% CS for 4 h at 38.5°C in a 5% CO₂ atmosphere, and then treated with 7% (v/v) ethanol in TCM199 supplemented with 1 mg/ml polyvinylpyrrolidone (Sigma-Aldrich) for 5 min as previously reported [27].

Embryo culture

The culture medium was modified synthetic oviduct fluid [28, 29] supplemented with 20 μ l/ml essential amino acid solution (50 \times Gibco BRL), 10 μ l/ml non-essential amino acid solution (100 \times Gibco BRL), 1 mM glycine, 2 mM taurine, ITS supplement (final concentrations of 5 μ g/ml insulin, 5 μ g/ml transferrin, and 5 ng/ml selenium; Sigma-Aldrich), and 6 mg/ml fatty acid-free BSA (Sigma-Aldrich). Sperm-injected oocytes were cultured in groups of 10–15 in 50 μ l drops of modified synthetic oviduct fluid medium at 38.5°C in a 5% CO₂, 5% O₂, and 90% N₂ atmosphere. Cleavage rates and blastocyst formation rates were assessed at 72 h and 192 h after ICSI.

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

The cell allocation of blastocysts (192 h after ICSI) was assessed by differential staining of the ICM and TE cells as described previously [30]. Briefly, blastocyst TE cells were stained for 40 sec with 100 μ g/ml propidium iodide (Sigma-Aldrich) in a permeabilizing solution of 0.2% (v/v) Triton X-100 (Sigma-Aldrich). Blastocysts were then counterstained and simultaneously fixed for 5 min with 25 μ g/ml Hoechst 33342 (Calbiochem, La Jolla, CA, USA) in 99.5% ethanol. Fixed and stained whole blastocysts were mounted, and the numbers of ICM and TE cells were assessed using fluorescence microscopy. ICM and TE nuclei were identified by blue and pink to red staining, respectively.

Labeling of bull spermatozoa with monobromobimane (mBBr)

Frozen spermatozoa straws from Japanese Black bulls were thawed at 37°C for 30 sec in a water bath washed with BSA-free mTALP [23], supplemented with 10 mM caffeine (Wako Pure Chemical Industries), and centrifuged at $500 \times g$ for 5 min [24]. Washed spermatozoa were then incubated with BSA-free mTALP supplemented with 8 mM GSH (Wako Pure Chemical Industries) for 10 min, 30 min, and 60 min at 38.5°C [12, 25, 26] and washed again. GSH was not added to the control group.

In a 1.5 ml microtube, mBBr (Calbiochem, La Jolla, CA, USA) was added at a final reagent concentration of 0.1 mM to the 1.0 ml sperm suspension ($4\text{--}5 \times 10^6$ sperm/ml) in mTALP. Sperm samples were covered with aluminum foil during the labeling reaction to minimize bimane photolysis. After 5 min incubation in darkness at 37°C, the sperm suspension was mounted on glass slides, covered with coverslips, and examined using fluorescence microscopy (Nikon, Tokyo, Japan) [31, 32]. Fluorescence emissions of sperm were recorded with a digital camera using a stabilized mercury lamp and fluorescence filters (excitation at 385 nm and emission at 484 nm) to determine emission values using Image J 1.48 software (National Institutes of Health, Bethesda, MD, USA).

Adenosine triphosphate (ATP) assay

The ATP content of sperm was measured with an ATP-dependent luciferin-luciferase bioluminescence assay (ATP Bioluminescent Assay kit; Sigma-Aldrich). Serial dilutions of the ATP standard ranging from 100 nM to 0.01 pM were prepared, and a standard curve was generated based on the relative light intensity of the serially diluted standard. The standard curve was then used to determine the ATP content of the samples. For each sperm sample preparation, 10×10^6 sperm cells from the control and GSH treatment groups were prepared. Two reaction vials were prepared per sample and 0.1 mL of ATP Assay Mix Solution was added. The mixture was incubated at room temperature (25–30°C) for 3 min, mixed, and the luminescence was measured immediately with a luminometer (Gene Light 55; Microtech, Chiba, Japan). Sample measurements were performed in duplicate and the mean was calculated.

Labeling of bull spermatozoa with Rhodamine 123 (R123)

The staining method used for sperm mitochondria was modified from Connell *et al.* [33]. Briefly, both sperm aliquots were incubated with R123 (Sigma-Aldrich) at a final ratio of 10 $\mu\text{l/ml}$ for 30 min at 37°C in a 5% CO₂ atmosphere. Excess R123 was removed by washing twice with mTALP medium and centrifuging at $500 \times g$ for 5 min. Sperm suspensions were mounted on glass slides, covered with coverslips, and examined with fluorescence microscopy (Nikon). Fluorescence emissions of sperm middle piece were recorded with a digital camera using a stabilized mercury lamp and fluorescence filters (excitation at 507 nm and emission at 529 nm). The fluorescence emission was analyzed with ImageJ 1.48 software.

Sperm motility assay

Sperm motility parameters were measured in the control and GSH treatment groups. Sperm were treated with GSH for 10 min and then washed. Subsequently, samples were incubated for 0 min, 60 min, 120 min, and 180 min in mTALP medium at 38.5°C in a

5% CO₂ atmosphere. Four microliters of the samples were placed onto 4-chamber slides with a depth of 12 μm (Leja, Nieuw-Vennep, Netherlands). At least 200 sperm cells across five fields per chamber were counted for motile and dead sperm, and both the percentage of motile sperm and sperm motility parameters were evaluated with a computer-aided sperm analyzer (CASA; Hamilton-Thorne, Beverly, MA, USA). The sperm parameters evaluated included total motility, progressive motility, straight line velocity, curvilinear velocity, amplitude of lateral head displacement, beat cross frequency, and linearity. [34].

Statistical analyses

Data from the mBBr staining of bull spermatozoa and the differential staining of ICM and TE cells were analyzed with one-way analysis of variance. Means were compared by Tukey's multiple comparison tests. Percentage data were analyzed with the chi-squared and Fisher's exact tests, and *t*-tests were used to analyze the sperm ATP concentration and mitochondrial function measurements. 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. P-values < 0.05 were considered statistically significant.

Results

Figure 1 shows the effect of GSH treatment on sperm disulfide bonds. We first counted sperm numbers at specific time points during GSH treatment (0 min, 30; 10 min, 44; 30 min, 38; and 60 min, 45). We then performed mBBr staining of the sperm and set the relative fluorescence intensity (RFI) produced by the head of the sperm at the treatment time of 0 min to 1.0. The RFIs at 10 min (2.03 ± 0.05 , mean \pm SEM) and 30 min (2.07 ± 0.09) were significantly higher ($P < 0.05$) than that at 0 min (1.00 ± 0.02), whereas the RFI at 60 min (2.78 ± 0.10) was significantly higher than that at any other treatment time ($P < 0.05$).

We next analyzed the effect of GSH treatment on the ATP concentrations of the sperm. Figure 2 shows the ATP concentrations in sperm treated with GSH for 10 min. The ATP concentration in the control group ($1.00168 \pm 0.03282 \times 10^{-8}$ mol/ 10^6 sp) was significantly lower ($P < 0.05$) than that in the GSH treatment ($1.22344 \pm 0.03870 \times 10^{-8}$ mol/ 10^6 sp).

Figure 3 shows the effect of GSH treatment on the sperm mitochondrial function based on R123 staining. We set the relative luminescence intensity (RLI) produced by the middle piece of the sperm in the control to 1.0. The GSH treatment (1.5 ± 0.2) exhibited a significantly higher RLI ($P < 0.05$) than that of the control group (1.0 ± 0.1).

The results of the motility assays of the control and GSH-treated spermatozoa using the CASA system are illustrated in Table 1. The GSH-treated group showed significantly higher ($P < 0.05$) motility at 120 and 180 min after GSH treatment than that of the control group. Similarly, the GSH-treated group (120 min after GSH treatment) showed higher progressive motility than that of the control group ($P < 0.0509$). No significant differences between the control and

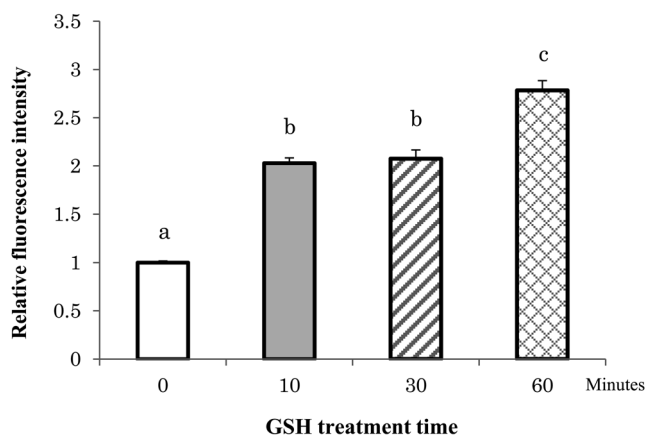


Fig. 1. Status of disulfide bonds in bovine sperm treated with glutathione (GSH). Sperm treated with GSH for 10 min, 30 min, and 60 min after sperm washing were stained with mBBR. The level of disulfide bonding is shown as the relative fluorescein intensity of monobromobimane (mBBR). Each column shows the mean \pm SEM (a-c: $P < 0.05$); one-way analysis of variance, and Tukey's multiple comparison test. The number of replicates is three.

GSH-treated groups were observed for the other motility parameters.

Table 2 shows embryo development after ICSI with GSH-treated sperm. No significant differences were observed in the total number of cleavages; however, the GSH-treated sperm showed significantly higher cleavages at the 4–8-cell stage than that of the untreated groups ($P < 0.05$). The percentage of blastocysts for GSH-ICSI without ethanol treatment at 192 h post-ICSI was also significantly higher ($P < 0.05$) than that of the Sp-ICSI with ethanol treatment and Sp-ICSI without ethanol treatments.

The numbers of cells in the blastocysts at 192 h following ICSI are shown in Table 3. GSH-ICSI without ethanol treatment resulted in significantly more ($P < 0.05$) TE cells and a significantly higher ($P < 0.05$) total cell number than that of the Sp-ICSI treatment.

Discussion

In the present study, we examined GSH treatment as a method of sperm pretreatment prior to bovine ICSI. Bovine sperm nuclei have particularly strong disulfide bonds [1], resulting in hyperstabilized chromatin that prevents decondensation of the sperm nucleus [35]. The protamine in bull sperm chromatin is a cysteine-rich type 1 protein, which results in a tightly packaged and stable chromatin configuration [36].

We investigated the influence of GSH on disulfide bond reduction in the sperm head, which was observed to increase with time in the GSH treatment. In a previous study, the use of DTT for sperm treatment has been discussed [37]. DTT showed a faster disulfide bond reduction rate than that of GSH, and the increase in disulfide bond reduction rate was gradually higher in the GSH treatment than that of the DTT treatment. However, DTT is known to have very strong reducing ability and chromosome abnormalities have been reported to occur in embryos produced by ICSI of DTT-treated sperm [16].

We initially thought that the influence of GSH treatment on embryo

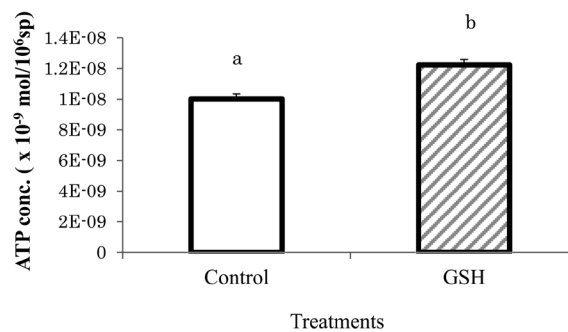


Fig. 2. Effect of function of bovine sperm mitochondria treated with GSH on adenosine triphosphate (ATP) concentration. The ATP content of sperm was measured as luminescence generated in an ATP-dependent luciferin–luciferase bioluminescence assay. Each column shows the mean \pm SEM (a, b: $P < 0.05$); *t*-tests. The number of replicates is three.

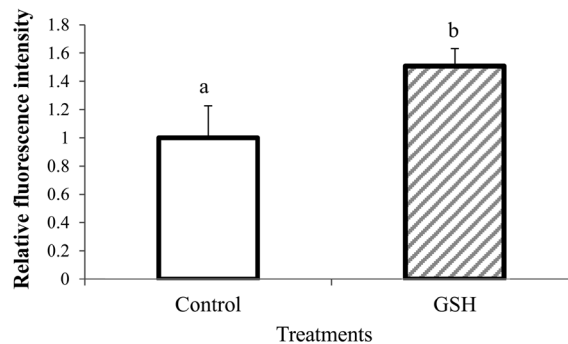


Fig. 3. Effect of function of bovine sperm mitochondria treated with GSH on Rhodamine (R123) staining. The level of mitochondria is shown as the relative fluorescein intensity of R123. Each column shows the mean \pm SEM (a, b: $P < 0.05$); *t*-tests. The number of replicates is three.

development would be less than that of DTT; however, GSH treatment resulted in rapid growth of 4–8-cell stage embryos. This is likely the result of GSH promoting embryo development from the 2-cell stage by effectively decreasing the level of disulfide bonding in the sperm. Therefore, although the disulfide bond reduction ability of GSH is lower than that of DTT, GSH remains an effective pretreatment for ICSI.

Previous studies have stated that DTT treatment of sperm decreases disulfide bonds in sperm protamine, resulting in decondensation of the sperm head [38–40]. Cheng *et al.* [41] used a sperm chromatin structural assay and a flow cytometer to measure the integrity of swine sperm chromatin following treatment with DTT or GSH. The DTT-treated sperm exhibited higher chromatin degeneration and single-stranded DNA than that of the GSH-treated sperm. These results indicate that DTT treatment might increase the susceptibility of sperm to chromosome abnormalities, which has been observed in other studies [41]. These authors also reported that GSH functions as a disulfide bond-reducing agent and promotes sperm chromatin decondensation *in vitro*, but with fewer side effects than that of

Table 1. Effect of GSH treatment on the motility of bovine spermatozoa

Sperm motility parameters	Treatment	Incubation time			
		0 min	60 min	120 min	180 min
Motile (%)	Control	45.7 ± 5.0	43.5 ± 2.5	41.5 ± 3.1 ^a	42.3 ± 0.8 ^a
	GSH	48.4 ± 3.5	50.5 ± 4.5	57.9 ± 1.0 ^b	61.8 ± 4.8 ^b
Progressive motility (%)	Control	16.8 ± 0.2	21.4 ± 0.8	11.7 ± 0.3	6.2 ± 1.0
	GSH	15.9 ± 1.7	21.3 ± 4.0	18.8 ± 2.5	11.0 ± 2.5
VSL (µm/sec)	Control	96.2 ± 0.3	89.8 ± 3.1	64.4 ± 3.5	52.1 ± 1.3
	GSH	84.8 ± 4.4	79.9 ± 5.1	68.1 ± 2.5	53.6 ± 3.0
VCL (µm/sec)	Control	256.2 ± 10.9	155.1 ± 9.2	163.0 ± 4.3	171.2 ± 5.9
	GSH	237.4 ± 8.7	142.8 ± 4.5	152.5 ± 4.0	161.9 ± 3.3
ALH (µm)	Control	10.0 ± 0.4	6.2 ± 0.5	6.8 ± 0.3	7.9 ± 0.5
	GSH	9.4 ± 0.3	6.6 ± 0.5	6.4 ± 0.2	7.6 ± 0.5
BCF (Hz)	Control	33.6 ± 1.6	39.2 ± 1.1	38.7 ± 1.0	37.2 ± 0.3
	GSH	32.7 ± 1.9	40.7 ± 1.7	37.6 ± 0.5	36.3 ± 0.5
LIN (%)	Control	40.0 ± 1.7	56.7 ± 0.9	40.0 ± 1.0	32.3 ± 0.9
	GSH	38.7 ± 1.5	52.7 ± 3.7	45.0 ± 2.6	34.7 ± 2.0

Data are shown as the mean ± SEM. Values with different superscript letters are significantly different (^{a, b}: P < 0.05); *t*-tests. The number of replicates is three. VSL = straight line velocity; VCL = curvilinear velocity; ALH = amplitude of head displacement; BCF = beat cross frequency; and LIN = linearity. In the GSH group, sperm were treated with 8 mM GSH for 10 min. Samples were cultured in mTALP medium at 38.5°C in a 5% CO₂ atmosphere.

Table 2. Effect of different sperm pretreatments on the *in vitro* development of bovine embryos generated by intracytoplasmic sperm injection (ICSI)

Pretreatments	Activation treatment	No. of oocytes	No. (%) of cleavages		No. (%) of blastocysts
			4-8 cell	Total	
GSH-ICSI	+	79	52 (65.8) ^a	62 (78.5)	17 (21.5) ^{ac}
GSH-ICSI	-	84	60 (71.4) ^a	63 (75.0)	26 (31.0) ^a
Sp-ICSI	+	76	38 (50.0) ^b	52 (68.4)	11 (14.5) ^{bc}
Sp-ICSI	-	71	26 (36.6) ^b	46 (64.8)	5 (7.0) ^b

Values with different superscript letters are significantly different (^{a-c}: P < 0.05). The number of replicates was three. Cleavage and blastocyst rates were evaluated 72 h and 192 h, respectively. Activation treatment was carried out with 7% ethanol for 5 min. Sp-ICSI, treatment only with mTALP before ICSI. GSH-ICSI, treatment with 8 mM glutathione.

Table 3. Effect of sperm pretreatments prior to ICSI in blastocysts on the total number of cells and numbers of inner cell mass and trophectoderm cells

Pretreatments	Activation treatment	No. of embryos	No. of cells		
			TE	ICM	Total
GSH-ICSI	+	15	109.2 ± 7.9 ^{ab}	38.9 ± 3.1	148.1 ± 8.0 ^{ab}
GSH-ICSI	-	20	121.6 ± 6.7 ^a	50.5 ± 3.5	172.1 ± 7.6 ^a
Sp-ICSI	+	10	91.9 ± 5.7 ^{bc}	39.2 ± 5.6	131.1 ± 7.4 ^b
Sp-ICSI	-	5	69.8 ± 10.6 ^c	46.0 ± 3.7	115.8 ± 10.0 ^b

Values with different superscript letters are significantly different (^{a-c}: P < 0.05). The number of replicates was three. Activation treatment was carried out with 7% ethanol for 5 min. Sp-ICSI, treatment only with mTALP before ICSI; GSH-ICSI, treatment with 8 mM glutathione.

DTT in ICSI [41].

Our study showed that the disulfide bond reduction rate in the sperm head was higher with the passage of time in the GSH treatment. GSH is an inherently superior sperm treatment reagent compared to

DTT because it occurs naturally *in vivo*. GSH is a major non-protein thiol compound in mammalian cells and is involved in many cellular functions, including amino acid transport, DNA and protein synthesis, reduction of disulfide bonds, and protection against oxidative stress.

The sulfhydryl group of GSH has been shown to confer protection against the cytotoxicity of oxidants, electrophiles, and free radicals [42]. From these studies, it is clear that GSH has a strong antioxidant effect and is required for the effective elimination of reactive oxygen species such as those generated during normal sperm processing. This provides insight into the possible mechanisms of how GSH positively effects embryonic development. However, the present study did not measure reactive oxygen, which should be further investigated in future studies.

In the case of normal fertilization, spermatozoa in which capacitation or the acrosome reaction has been induced become decondensed after entering the oocyte. Sperm decondensation *in vivo* requires two major steps, S-S to S-H reduction in the protamines, followed by replacement of protamines with histones [3]. GSH is also present in the oocyte cytoplasm as a reducing agent for endogenous disulfide bonds and helps to protect the oocyte against oxidative stress [1]. GSH is used directly as an inducer of sperm decondensation *in vitro* in humans [43], cows [44], and hamsters [45].

The addition of GSH also helps maintain the motility of cryopreserved livestock sperm [46–50] by protecting sperm from oxidative damage [51]. In the present study, the concentration of ATP, which is an essential energy source for sperm tail movement, was measured. GSH-treated sperm showed high ATP concentrations and mitochondrial function, and sperm motility was maintained for extended periods. In the control group sperm, sperm treatment processes such as freezing and thawing affected the mitochondria and motility. This might be attributed to a reduction in ATP concentration over time. Soderquist *et al.* [52] showed a significant correlation between ATP concentration and sperm motility in bovine semen. R123, which is used to detect mitochondrial function, accumulates in the mitochondria by permeating the cell membrane [52]. Therefore, the accumulated fluorescence intensity of R123 reflects the amount of ATP production in the mitochondria. The present study is the first to report on the association between GSH treatment and ATP levels in bovine sperm.

The GSH-treated sperm also showed superior maintenance of sperm motility over extended periods. Gogol *et al.* [53] examined the ATP concentration and motility of rabbit sperm and reported that the total sperm motility ratio and progressive sperm motility were strongly correlated with ATP content. The present study showed comparable results.

The ICSI of GSH-treated sperm exhibited a significantly increased blastocyst development rate and embryonic development rate at the 4–8-cell stage. In addition, the total number of cells in the blastocysts significantly increased. This suggests that GSH treatment of sperm promotes embryo development and results in the production of high quality embryos. These results were similar to those observed for embryo development by ICSI using DTT-treated sperm published in a previous study [37]. Both reagents promote disulfide bond reduction; however, GSH appears to also maintain high sperm ATP concentrations. Bovine ICSI usually requires oocyte activation after sperm injection. Fujinami *et al.* [54] reported that ethanol treatment after ICSI temporarily inhibited maturation promoting factor activity 6 h after ICSI, and thus promoted subsequent embryonic development. We obtained good results from ethanol treatment for 4 h following ICSI [22]; however, these results indicate that there is no need for treatment with ethanol when sperms are pretreated with GSH.

This is similar to our previous study using DTT [37]. The precise mechanism behind this finding is the subject of future investigation. A previous study reported that piezo-ICSI using tail-cut motile spermatozoa is effective for cleavage and subsequent development without exogenous oocyte activation, which resulted in the birth of five calves [55]. This shows that oocyte activation factors in bovine spermatozoa are sufficient for embryo development. Bovine ICSI without additional activation treatment is important for achieving high rates of production in healthy calves.

Sekhavi *et al.* [56] reported three degrees of nuclear decondensation in male gametes with a combined pretreatment of heparin-glutathione for 7 h. Similarly, Zambrano *et al.* [57] discussed that treatments with a shorter incubation time reached a nuclear decondensation similar to degree. These studies, unlike our results, showed that long incubation times did not affect embryonic development after ICSI with heparin-glutathione [56, 57]. We demonstrated that the optimal GSH alone treatment time was 10 min and observed the reduction of disulfide bonds in the Japanese Black bull sperm head with the GSH alone treatment. Future studies that adjust incubation times and use different dose of reducing agents are required to improve the sperm nuclear decondensation in the efficiency of this technique in the bovine.

In conclusion, GSH treatment of bovine sperm decreased disulfide bonds in the head of the sperm and activated mitochondrial function in the sperm tail. The blastocyst developmental rate was shown to increase and the production of high quality embryos occurred using this method.

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