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Original Article

Effects of reduced glutathione supplementation in semen freezing extender on frozen-thawed bull semen and *in vitro* fertilization

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Abstract. During cryopreservation, spermatozoa may suffer cold and cryo-induced injuries —associated with alterations in cell defense systems— that are detrimental to their function and subsequent fertility. This study aimed to determine the efficacy of supplementing the semen freezing extender with the antioxidant reduced glutathione (GSH) in cattle. Semen was collected from four bulls and diluted in a freezing extender supplemented with or without GSH (0, 1, 5, and 10 mM) before the cooling step of the cryopreservation process. After thawing, the quality of the frozen-thawed semen was investigated for motility, viability, acrosomal and DNA integrity, and subsequent embryo development after *in vitro* fertilization of bovine oocytes. Additionally, semen from one of the bulls was used to analyze semen antioxidative potential, sperm penetration into oocytes, male pronucleus formation rate, and embryo DNA integrity. The sperm quality varied among bulls after GSH supplementation. One bull had decreased sperm total motility, and two bulls had decreased sperm DNA integrity. GSH supplementation had positive effects on embryo development for three bulls. Two of them showed both improved cleavage and blastocyst formation rates, while the other one only showed an improved cleavage rate. We observed positive effects on early male pronucleus formation and no negative effects on DNA integrity and cell number in blastocyst stage embryos. Although the effect varies depending on individual bulls and GSH concentration, GSH supplementation in semen may improve *in vitro* embryo production from frozen semen.

Key words: Bull, Frozen-thawed semen, Reduced glutathione (GSH), Semen characteristics, Semen freezing extender

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The utilization of frozen semen obtained from bulls with excellent genetic traits has been highly effective in increasing profitability and achieving genetic improvement owing to efficient calf production. In addition, the recent development of sperm sexing technology [1] has further heightened the practical benefit of frozen semen by allowing the breeders to obtain calves of the desired gender. However, conception rates after artificial insemination in cattle have been gradually declining over the recent decades in Japan and other countries [2, 3]. Generally, frozen-thawed semen has a lower quality manifested as decreased viability [4–6], motility [5–7], acrosome integrity [6–8], plasma membrane integrity [4, 6], and DNA integrity [4, 8] compared to the fresh semen. Cryopreservation-related injuries during the freezing and thawing processes decrease cellular metabolism, alter membrane permeability, and induce the loss of intracellular components [9]. These injuries are associated with excess production of reactive oxygen species (ROS) and a change in antioxidant defense systems in the cells [9].

In spermatozoa, ROS are generated metabolically and are necessary for sperm function [10]. However, excess levels of ROS cause oxidative damage, such as membrane lipid peroxidation and DNA fragmentation that adversely affect early embryo and subsequent fetal development after fertilization [11, 12]. Supplementing the freezing medium with antioxidants has been used in several species to reduce the detrimental effects of ROS on spermatozoa [13]. Reduced glutathione (l-γ-glutamyl-l-cysteinyl-glycine: GSH) is a biological antioxidant ubiquitously distributed in living cells that plays an important role in intracellular defense against oxidative stress [14]. GSH can act both as an antioxidant, using the reducing power of its sulfhydryl group, and as a cofactor for antioxidant enzymes. The cryopreservation process reduces the GSH content in spermatozoa from cattle [5, 15], pigs [16], and humans [17]. GSH supplementation in an egg yolk-based semen freezing extender (SFE) has been tested in several bovine breeds under different conditions [6, 8, 18–20]. However, the effect of GSH supplementation and effective concentration on different breeds and individuals of other mammalian species previously reported [21, 22] are not fully understood in bovine semen. Therefore, the present study focused on the individual differences in cattle semen quality to better understand the effects of GSH supplementation in SFE on frozen-thawed semen and the subsequent quality of embryos fertilized *in vitro*.

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Materials and Methods

Reagents

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise.

Preparation of frozen semen with or without GSH-supplemented SFE

Fresh semen was collected from four bulls: one Holstein bull (Bull 1) and three Japanese Black bulls (Bulls 2–4). Each semen sample was frozen to evaluate its quality and used in the *in vitro* fertilization (IVF) procedure (Supplementary Table 1). The animal experiments were approved by the Committee for the Care and Use of Experimental Animals at NILGS (No.1611BO11). Semen samples from these bulls were frozen, as previously reported [23]. Each ejaculate was divided into four equal portions and diluted with the first SFE to obtain a final (frozen step) concentration of 0, 1, 5, or 10 mM GSH. After equilibration at 4°C, an equal volume of the second SFE containing glycerol was added to obtain a final concentration of 6.5% glycerol. The final concentrations of diluted semen samples were 0.7–3.4 × 10^8 (Bull 1), 0.8–3.2 × 10^8 (Bull 2), 0.2–0.4 × 10^8 (Bull 3), and 0.5–3.2 × 10^8 (Bull 4) spermatozoa/ml. The osmolarity of SFE was measured using a vapor pressure osmometer (VAPRO 5520, Wescor, UT, USA) (Supplementary Table 2).

For sperm quality evaluation and IVF, frozen semen samples were thawed in a water bath at 38°C for 20 sec and were subjected to analysis and IVF protocols as described below.

Analysis of sperm motility

To evaluate sperm motility, frozen-thawed sperm aliquots from each group were diluted five-fold with Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride (PBS (–)). Samples from four bulls (three to eight semen lots) were then analyzed respectively using a computer-assisted sperm analysis (CASA) system (SMAS for Animal sperm, DITECT, Tokyo, Japan). For each measurement, a 10 µl aliquot was loaded onto a chamber (Makler counting chamber, Fujifilm Wako, Tokyo, Japan) at a depth of 10 µm. Approximately 900 spermatozoa from three videos taken at 60 frames per second were analyzed for each specimen. The following variables were analyzed: total percentage of motile sperm (MOT, %), average path velocity (VAP, µm/sec), straight-line velocity (VSL, µm/sec), curvilinear velocity (VCL, µm/sec), amplitude of lateral head displacement (ALH, µm), beat/cross frequency (BCF, Hz), straightness (STR = VSL/VAP × 100, %), linearity (LIN = VSL/VCL × 100, %), and wobble (WOB = VAP/VCL × 100, %). Data are presented as mean ± standard error for each group.

Evaluation of sperm acrosomal integrity and viability

The acrosome integrity of frozen-thawed spermatozoa was evaluated using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC–PNA)/propidium iodide (PI) staining [24] (Fig. 1A). Samples from four bulls (three to four semen lots) were diluted respectively by 10-fold using FITC–PNA solution (final concentration: 1.0 µg/ml) to assess the acrosomal status and PI solution (final concentration: 6.0 µM) to assess the viability at 2 h after thawing, and then incubated for 20 min at 37°C. The unbound probes were removed by washing with PBS (–), and the precipitated spermatozoa were resuspended for mounting on a slide. The observations were performed under a fluorescence microscope (TE2000U, Nikon, Tokyo, Japan) at a magnification of 200 × using a B-2A filter (450–490 nm excitation filter, 505 nm dichroic mirror, 520 nm bandpass filter) for FITC–PNA and a G-2A filter (510–560 nm excitation filter, 575 nm dichroic mirror, 590 nm bandpass filter) for PI. Approximately 400 spermatozoa were observed on each prepared slide from five fields of vision. The viability and acrosome integrity were determined by counting PI (–) (Fig. 1A, b and c) or FITC–PNA (−) (Fig. 1A, b and d) spermatozoa per total spermatozoa. The acrosome integrity of living spermatozoa was determined by counting FITC–PNA (−) spermatozoa (Fig. 1A, b)

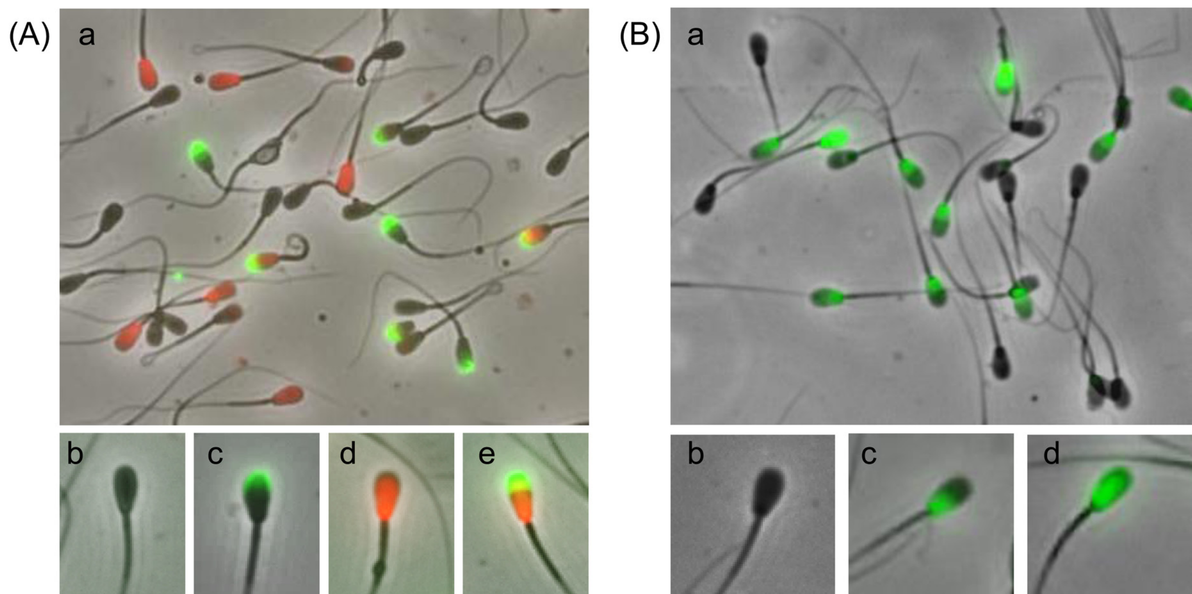

Fig. 1. Classification of spermatozoal integrity using staining methods. (A) Viability and acrosome integrity of bull spermatozoa evaluated with FITC-PNA/PI staining. a, Overall picture; b, FITC–PNA$^-$/PI$^-$ (acrosome intact, live); c, FITC–PNA$^+$ (damaged acrosome, live); d, PI$^+$ (acrosome intact, dead); e, FITC–PNA$^+$/PI$^+$ (damaged acrosome, dead). (B) DNA fragmentation status of bull spermatozoa evaluated using TUNEL assay. a, Overall picture; b, TUNEL$^-$ (–, unfragmented DNA); c, TUNEL-partially positive (±; fragmented DNA); d, TUNEL-entirely positive (+; fragmented DNA). The image of phase–contrast and fluorescence picture were merged for observation.

per PI (−) spermatozoa. Data are presented as mean ± standard error for each group, and three to four replicates were used for each group.

Evaluation of sperm DNA integrity

Sperm DNA integrity was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, as described by Takeda *et al*. [25] (Fig. 1B). Fresh semen (four to six semen lots) was diluted using PBS (–), and frozen-thawed semen (from the same lots as fresh samples) was washed and diluted with PBS (–). Both semen samples were diluted to adjust the sperm concentration to 0.2×10^8 spermatozoa/ml. Diluted samples (15 µl) were fixed onto the slides using 2% paraformaldehyde and washed with PBS (–). Samples were permeabilized with 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 2 min on ice and then incubated in TUNEL reaction mixture from a commercial kit (In Situ Cell Death Detection Kit, fluorescein, Roche, Indianapolis, IN, USA) for 1 h at 37°C in a dark and humidified atmosphere. Positive TUNEL staining was observed under a fluorescence microscope (TE2000U, Nikon) using a B-2A filter. Approximately 800 spermatozoa were observed on each prepared slide from five fields of vision. Sperm DNA damage, expressed as DNA fragmentation index (DFI), was determined by the ratio of the entirely positive (+) (Fig. 1B, d) and partially positive (±) (Fig. 1B, c) stained spermatozoa. Data are presented as mean ± standard error for each group.

Measurement of semen antioxidative status

Frozen-thawed samples obtained from Bull 1 and 2 were used to evaluate antioxidative status. Fifty µl of each sample ($0.8–1.9 \times 10^8$ spermatozoa/ml, one lot) was used for the biological antioxidant potential (BAP) test conducted by the analyzing company (Wismerll Co., Ltd., Tokyo, Japan). The BAP test is based on the ferric reducing ability of the plasma [26]. Samples were dissolved in a colored solution, initially prepared by mixing $FeCl_3$ with a thiocyanate derivative. After incubation, the intensity of chromatic change that is directly proportional to the ability of the samples to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), was photometrically read.

The levels of lipid peroxidation were assessed by determining malondialdehyde (MDA) production using a thiobarbituric acid reactive substances (TBARS) assay kit (OxiSelect™ TBARS Assay Kit, Cell Biolabs, CA, USA). Frozen-thawed samples from Bull 1 and 2 (four semen lots) were used for the TBARS assay. To avoid interference by the egg yolk, the extender was removed by centrifugation (700 × g, 5 min, 4°C). The pellets were resuspended in PBS (–) to adjust the sperm concentration to 1×10^8 spermatozoa/ml, followed by the addition of 1 × butylated hydroxytoluene. Further, the samples or MDA standards were mixed with a sodium dodecyl sulfate lysis solution. After 5 min of incubation, the thiobarbituric acid reagent was added, followed by incubation at 95°C for 60 min. Cooled and centrifuged supernatants were used for measurements. The level of fluorescence at 540 excitation/590 emission was determined using a microplate reader (GENios, Tecan, Maennedorf, Switzerland). The results are presented as µM MDA/ 10^8 spermatozoa.

IVF with frozen-thawed semen

IVF was performed in two laboratories—NILGS for Bull 1 and 2 and HLTRC for Bull 3 and 4. To examine the effect of different concentrations of GSH, 1 mM, 5 mM, and 10 mM (Bull 1: five lots, and Bull 2: four lots), and 5 mM and 10 mM (Bull 3 and 4, three lots) were used for IVF. Oocyte collection, *in vitro* maturation (IVM), IVF, and *in vitro* culture (IVC) were performed as described previously at NILGS (Bull 1 and 2) [27] and HLTRC (Bull 3 and 4) [28]. Briefly, bovine ovaries were obtained from a slaughterhouse and stored in PBS (–) with antibiotics until oocyte collection. Cumulus-oocyte complexes (COCs) were collected, and groups of 40–70 COCs were cultured for 22–24 h at 38.5°C under 5% CO_2 in air. Matured oocytes were then co-cultured with washed sperm in 100 µL droplets of IVF medium ($0.05–0.06 \times 10^8$ spermatozoa/ml) for 6 h at 38.5°C under 5% CO_2 in air (10–20 oocytes per droplet). At the end of insemination, putative zygotes were completely denuded from cumulus cells and spermatozoa by gentle pipetting. IVC was performed in 200 µl droplets of IVD 101 medium (IFP, Yamagata, Japan) (Bull 1 and 2) or 50 µl droplets of modified m-SOF medium (Bull 3 and 4). Subsequently, 10–20 zygotes were placed in each culture drop and then cultured at 38.5°C under 5% O_2 and 5% CO_2 in air for 8–9 days (the day of IVF was considered to Day 0). Cleavage rates in each treatment group were recorded on Day 2 or 3. The rates of embryos developing to the blastocyst stage were recorded on Days 6–9.

Assessment of fertilization status

Fertilization status regarding sperm penetration and PN (pronucleus and pronuclei) formation was evaluated at 9 h and 18 h after IVF (the insemination timing, introducing sperm to oocytes considered to 0 h). Oocytes inseminated for 6 h with the semen samples from Bull 1 (presumptive zygotes: PZ) were used in the assay as previously described [29] with some modifications. Briefly, the PZ were randomly picked from each IVC drop, mounted on glass slides, and fixed in a mixture of acetic acid and ethanol (1:3) for at least 3 days. The fixed PZ were then stained with 1% (w/v) orcein in acetic acid, rinsed in a mixture of glycerol, acetic acid, and water (1:1:3), and then examined under a phase–contrast microscope (Axioskop, ZEISS, Oberkochen, Germany). The presence and number of male and female PN and/or sperm head(s) were then investigated in reference to a previous report [30]. Spermatozoa were considered to have the ability to penetrate and fertilize the oocytes when a male PN and/or sperm heads with attached sperm tails were observed inside the PZ, which were then classified as fertilized. The presence of both one sperm head and female chromosome or one male and one female PN indicated normal fertilization. The presence of more than two sperm heads or male PNs indicated polyspermy. Spermatozoa were considered to have the ability to decondense and form male PN when male PN was present in the cytoplasm of the PZ. Only male PN that had reached an enlarged size was added up to the number of male PN formations.

Evaluation of embryo quality

IVF embryos obtained from Bull 1 were assessed for embryo quality, represented as developmental competence and DNA integrity, at the expanded blastocyst stage on Day 7 or 8. The TUNEL assay was performed on the embryonic nuclei, combined with differential staining of inner cell mass (ICM) and trophectoderm (TE) cells, as previously described [31] with slight modifications. Embryos were partially permeabilized in 0.2% (v/v) Triton X-100 in PBS (–) and 0.02 mg/mL polyvinylpyrrolidone (PVP) solution (PVP-PBS). TE cells were stained with 30 µg/mL PI in PVP-PBS. Embryos were fixed and stained in PVP-PBS containing 4% (w/v) paraformaldehyde and 10 µg/ml Hoechst 33342. Embryos were then permeabilized in 0.1% (w/v) sodium citrate containing 0.1% (v/v) Triton X-100. After washing in PVP-PBS, the embryos were incubated in the TUNEL reaction mixture. Observations were performed under UV light with excitation at 365/10 nm and emission at 400 nm using an epifluorescence microscope (Eclipse E800; Nikon). A digital image of total cell number (DNA), TE cells, and TUNEL-labeled nuclei for each embryo was captured (NIS-Elements BR ver.4.30, Nikon), and

the three images were composited using Adobe Photoshop Elements 2019 software. The number of cells and TUNEL-labeled nuclei were considered as an index of DNA damage and were determined using NIH ImageJ (v. 1.52a) software [32]. Data are presented as mean ± standard error, and 14–19 embryos were used for each group.

Statistical analysis

Differences among the GSH treatment groups for each bull (Fig. 2, Supplementary Tables 3 and 4) and differences among bulls in the 0 mM GSH control groups (Fig. 2, Supplementary Table 3) were analyzed by one-way analysis of variance (ANOVA) with the Tukey-Kramer test. Differences in DFI among GSH treatment groups in each bull and individual differences among bulls in fresh and 0 mM GSH control groups were analyzed using the Kruskal-Wallis test, followed by Dunn's test (Fig. 3). Fertilization rates and corresponding fertilization status after IVF for each GSH concentration (Table 1),

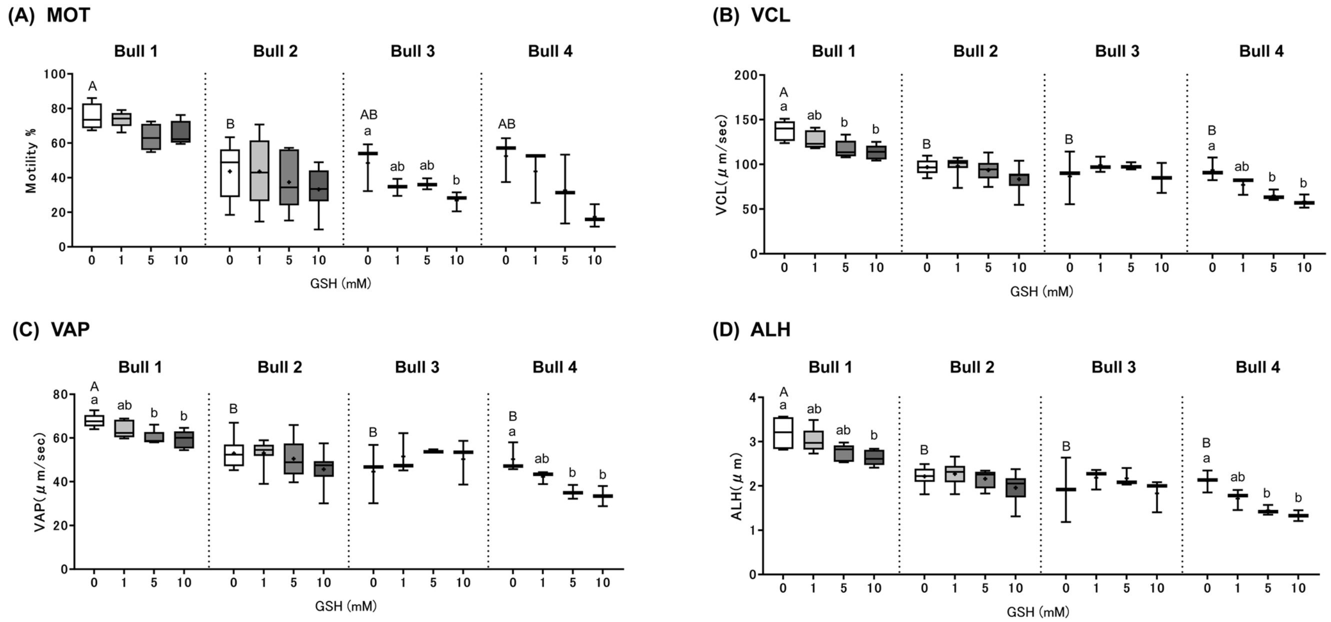

Fig. 2. Effects of GSH supplementation on sperm motility in four bulls. Box plots represent (A) total motility (MOT, %), (B) curvilinear velocity (VCL, µm/sec), (C) average path velocity (VAP, µm/sec), and amplitude of lateral head displacement (ALH, µm) of frozen-thawed spermatozoa (n = 3–8 samples). Significant differences ($P < 0.05$) are represented by small letters (a, b) for differences among GSH concentrations for each bull and capital letters (A, B) for differences among bulls in the 0 mM GSH control group.

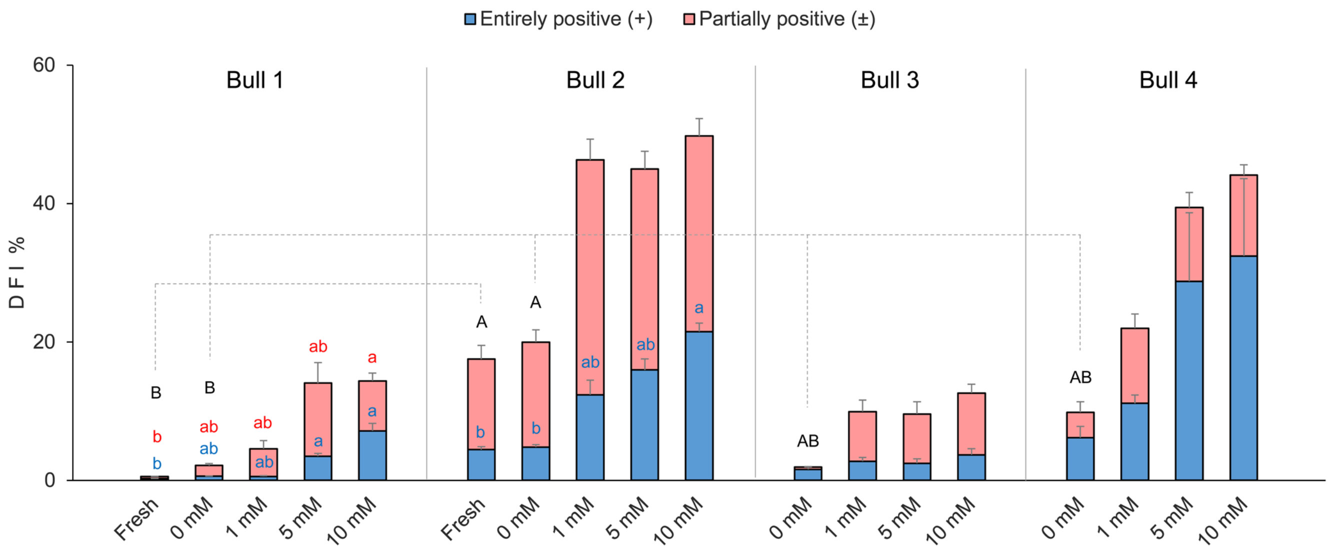

Fig. 3. Effects of GSH supplementation on sperm DNA integrity (DNA fragmentation indices (DFI)) in fresh and frozen-thawed semen samples from four bulls. Fresh semen (Bull 1, n = 4; Bull 2, n = 6) and frozen-thawed semen (Bull 1, n = 4; Bull 2, n = 6; Bull 3, n = 3; Bull 4, n = 3) samples were evaluated for DFI using the TUNEL assay. DFI is represented as a percentage of TUNEL^{+} spermatozoa, with entirely (+, blue bar) or partially (±, red bar) positive fluorescent heads indicating fragmented DNA (Fig. 1b) and mean ± SE. Different letters indicate significant differences among the GSH concentrations in each bull ($P < 0.05$) as follows: a–b with blue: entirely positive sperm (%) and a-b with red: partially positive sperm (±). The differences in entirely positive DFI among bulls in fresh and 0 mM GSH control groups were analyzed, and significant differences were detected between Bull 1 and 2 (A–B: $P < 0.05$).

the developmental stages of blastocysts in each bull, and differences among bulls at 0 mM GSH (Table 2) were analyzed using the Chi-square test. Embryo quality at each GSH concentration was analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney test (Table 3). Differences were considered statistically significant at $P < 0.05$. All statistical analyses were performed using R statistical software (version 3.6.1 and 4.0.2).

Results

Quality of frozen-thawed semen prepared with GSH-supplemented SFE

We measured the osmolality of fresh semen, control SFE, and GSH-SFE (GSH-supplemented SFE) to assess the physical impact on spermatozoa. Even though the sperm in GSH-SFE were exposed to double concentrations of GSH in the first dilution step, the osmolality was in the normal range (309.0–342.7 mmol/Kg), similar to that of the fresh semen (342.0–366.0 mmol/Kg) and control groups (300.0 mmol/Kg) (Supplementary Table 2).

The motility characteristics of frozen-thawed spermatozoa prepared with or without GSH-SFE were assessed using CASA. There were individual differences in five motility parameters (MOT, VCL, VAP, ALH, and WOB) in the initial scores of the 0 mM GSH control group (Fig. 2, $P < 0.05$, WOB: differences between Bull 1 (0.5%) and Bull 2 (0.6%)). In the case of GSH-SFE, there were no significant changes in motility parameters except MOT, VCL, VAP, and ALH (Fig. 2, $P < 0.05$). In all four bulls, increasing GSH concentration in SFE had an inversely affected MOT but, this relationship was only significant in Bull 3 with 10 mM GSH-SFE compared to the 0 mM GSH controls (Fig. 2A). A similar and significant inverse relationship between increasing GSH concentration and the other three motility parameters was also observed in Bull 1 with 10 mM GSH-SFE and Bull 4 in 5 and 10 mM GSH-SFE compared to 0 mM GSH controls (Fig. 2B, C, D). No significant differences were observed for Bull

Table 1. Fertilization status of IVF embryos derived from frozen-thawed semen prepared with GSH-supplemented semen freezing extender

GSH concentration	No. of oocyte (% total)	No. of oocyte (% penetrated)			
	Sperm penetration	Normal fertilization	Polyspermic fertilization	Male PN formation	
				9 h after IVF	18 h after IVF
0 mM	93 / 113 (82.3)	65 / 93 (69.9)	27 / 93 (29.0)	17 / 41 (41.5) [b]	50 / 52 (96.2)
1 mM	66 / 83 (79.5)	42 / 66 (63.6)	21 / 66 (31.8)	18 / 25 (72.0) [a]	40 / 41 (97.6)
5 mM	91 / 108 (84.3)	56 / 91 (61.5)	31 / 91 (34.1)	26 / 32 (81.3) [a]	58 / 59 (98.3)
10 mM	75 / 90 (83.3)	49 / 75 (65.3)	25 / 75 (33.3)	34 / 40 (85.0) [a]	34 / 35 (97.1)

Frozen-thawed semen samples from Bull 1 were subjected to a fertilization status test. PN, pronuclei, and pronucleus. Samples were fixed at 9 h (three replicates) and 18 h (eight to ten replicates) after IVF. Spermatozoa were considered to have the ability to penetrate and fertilize when male PN and/or sperm heads with the contributing sperm tails were observed inside the oocyte. The presence of one sperm head and female chromosome or one male and one female PN were considered to indicate normal fertilization. Two or more sperm heads and/or male PNs were considered to indicate polyspermy. Spermatozoa were considered to have the ability to decondense and form male PN when enlarged male PN was present in the cytoplasm.

Table 2. *In vitro* development of IVF embryos derived from frozen-thawed semen prepared with GSH-supplemented semen freezing extender

Bull	GSH concentration	Total number (No.) of oocytes cultured	No. of developed embryos (%)	
			Cleaved	Blastocytes
1	0 mM	267	173 (64.8) [b A]	63 (23.6) [A]
	1 mM	245	177 (72.2) [b]	69 (28.2)
	5 mM	288	188 (65.3) [b]	84 (29.2)
	10 mM	264	212 (80.3) [a]	66 (25.0)
2	0 mM	237	45 (19.0) [b C]	16 (6.8) [b B]
	1 mM	265	86 (32.5) [a]	28 (10.6) [ab]
	5 mM	182	61 (33.5) [a]	24 (13.2) [a]
	10 mM	273	111 (40.7) [a]	42 (15.4) [a]
3	0 mM	154	114 (74.0) [A]	49 (31.8) [A]
	1 mM	ND	ND	ND
	5 mM	152	117 (77.0)	50 (32.9)
	10 mM	131	97 (71.7)	40 (30.5)
4	0 mM	240	136 (56.7) [b B]	63 (26.3) [b A]
	1 mM	ND	ND	ND
	5 mM	225	176 (78.2) [a]	93 (41.3) [a]
	10 mM	78	57 (71.7) [a]	66 (32.2) [ab]

Data from 3–9 replicates. Values with different superscripts within the same column of each bull (a–b: $P < 0.05$) and among bulls in the 0 mM GSH control group (A–C: $P < 0.05$) were significantly different. ND = not determined.

Table 3. Quality of blastocysts derived from IVF with frozen-thawed semen prepared with GSH-supplemented semen freezing extender

GSH concentration	No. of embryo	No. of cell			ICM/Total (%)	No. of cells with damaged DNA (TU)		
		ICM	TE	Total		TU-ICM (TU-ICM/ICM%)	TU-TE (TU-TE/TE%)	TU-Total (TU-Total/Total%)
0 mM	19	33.7 ± 2.9 [b]	115.7 ± 15.1	149.4 ± 16.1	24.8 ± 2.0 [b]	1.5 ± 0.3 [a] (4.5 ± 1.1) [a]	5.4 ± 0.9 (5.5 ± 1)	6.7 ± 0.9 (5.1 ± 0.8)
1 mM	14	43.4 ± 3.4 [a]	112.4 ± 14.3	155.8 ± 16	29.6 ± 2.2 [ab]	0.8 ± 0.4 [ab] (1.8 ± 0.9) [b]	5.1 ± 1.0 (5.5 ± 1.4)	5.9 ± 1.1 (4.1 ± 0.8)
5 mM	18	44.0 ± 4.6 [ab]	100.5 ± 10.9	144.5 ± 13.5	31.3 ± 2.1 [a]	1.5 ± 0.4 [ab] (3.1 ± 0.9) [ab]	5.3 ± 1.1 (5.3 ± 1.1)	6.7 ± 1.3 (4.5 ± 0.8)
10 mM	14	42.0 ± 4.6 [ab]	96.9 ± 7.4	138.9 ± 8.4	30.5 ± 2.7 [ab]	0.6 ± 0.3 [b] (1.2 ± 0.5) [b]	4.0 ± 1.0 (4.1 ± 0.8)	4.6 ± 1.1 (3.2 ± 0.6)

Frozen-thawed semen samples from Bull 1 were subjected to embryo quality evaluation. Six to ten replicates were used. Values are expressed as mean ± SE. ICM, inner cell mass; TE, trophectoderm; TU, TUNEL-positive. a–b: Different superscripts in the same parameter denote significant differences (P < 0.05) among the GSH concentration groups.

2 semen. In total, there were no significant changes in motility or velocity of frozen-thawed semen prepared with 1 mM GSH-SFE but, a decrease in these characteristics was observed in the semen from three bulls (Bull 1, 3, and 4) with 10 mM GSH-SFE.

We analyzed the viability and acrosome integrity using FITC–PNA/PI staining (Supplementary Table 3). No individual differences among bulls were detected in the 0 mM control group. There were no significant differences in the percentage of viable spermatozoa ($PI^–$), intact acrosome ($FITC–PNA^–$), or viable spermatozoa with intact acrosome ($FITC–PNA^–/PI^–$) at any GSH concentrations in all bulls.

Sperm DNA integrity was also assessed using the TUNEL assay (Fig. 3). We assessed the initial differences in DFI among bulls and detected significant differences between Bull 1 and 2 in fresh (entirely (+) or partially (±) positive) and 0 mM GSH control (entirely positive, +) groups. Individual differences in DFI with increasing GSH concentration in SFE were observed in frozen-thawed samples among bulls. In Bull 1, the sperm DFI (entirely (+) or partially (±) positive) was higher in the 5 mM and 10 mM GSH-SFE groups than in their fresh semen counterparts (P < 0.05). In Bull 2, the DFI (entirely positive, +) at 10 mM GSH-SFE was higher than that of the fresh and 0 mM GSH-SFE (P < 0.05). There were no significant effects of GSH supplementation in SFE on sperm DNA integrity in Bull 3 and 4. The DFI (entirely positive, +) in Bull 2 and 4 showed a considerable increase (>20%) at 5 or 10 mM GSH supplementation. Overall, DFI increase due to high GSH supplementation concentration was observed, but the trend varied among the bulls.

We performed the BAP and TBARS assays to analyze the antioxidative status of frozen-thawed semen prepared with GSH-SFE. There was no difference in BAP and MDA levels among the different samples with GSH supplementation at different concentrations (Supplementary Table 4).

Taken together, GSH-SFE does not seem to have significant negative effects on frozen-thawed semen quality, except at high concentrations in some bulls.

IVF using frozen-thawed semen prepared with GSH-supplemented SFE

We evaluated the capacity for fertilization of semen prepared with GSH-SFE at 9 h and 18 h after IVF (Table 1). Data from Bull 1 showed three decreased motility parameters (Fig. 2) and increased DFI (Fig. 3) due to GSH supplementation. There were no significant differences in penetration rates, normal or polyspermic fertilization rates, and the male PN formation rates at 18 h after IVF, regardless of GSH concentration. The male PN formation rates evaluated at 9 h after IVF were significantly higher in the 1 mM (P < 0.05), 5 mM (P < 0.01), and 10 mM (P < 0.01) GSH-SFE groups than in the 0 mM GSH control.

The cleavage and blastocyst formation rates of IVF embryos derived from frozen-thawed semen prepared with or without GSH-SFE are shown in Table 2. At 0 mM GSH, there were individual differences among bulls in the cleavage and blastocyst formation rates (P < 0.05). GSH-SFE had positive effects on embryo cleavage and blastocyst formation in Bull 1, 2, and 4. Cleavage rates were significantly higher in embryos derived from 1 mM GSH-SFE in Bull 2 (P < 0.01), 5 mM GSH-SFE in Bull 2 and 4 (P < 0.01), and 10 mM GSH-SFE in Bull 1, 2, and 4 (P < 0.01) compared with the 0 mM GSH control. The cleavage rates of embryos derived from 10 mM GSH-SFE were higher than those from 1 mM and 5 mM GSH-SFE in Bull 1 (P < 0.05); however, cleavage rates were unaffected by GSH concentrations in Bull 2, 3, and 4. Increased blastocyst formation rates were observed in Bull 2 and 4 in embryos derived from GSH-SFE. In Bull 2, the highest blastocyst rate was observed in embryos derived from 10 mM GSH-SFE, whereas in Bull 4, 5 mM GSH-SFE resulted in the highest blastocyst rate. In Bull 1 and 3, there were no significant differences in blastocyst formation rates among the different GSH supplementation groups. Overall, GSH-SFE did not have any detrimental effects on embryo development, while its positive effect varied among bulls.

We then evaluated the quality of the IVF blastocysts derived from the frozen-thawed semen of Bull 1 based on the total cell number, relative ICM cell proportion, and the integrity of the DNA in the blastocyst cells (Table 3). The number of ICM cells in blastocysts derived from 1 mM GSH-SFE and the percentage of ICM/total cells in blastocysts derived from 5 mM GSH-SFE were significantly higher than those from the 0 mM GSH controls (P < 0.05). Blastocysts derived from 10 mM GSH-SFE had a lower DNA damage index in ICM cells compared with the 0 mM GSH controls. There was no significant difference among the GSH supplementation groups in the number or proportion of TUNEL-positive cells in TE and total cells. GSH-SFE increased the number of ICM cells and decreased the index of DNA damage in ICM cells of IVF blastocysts at different concentrations.

Discussion

In this study, by supplementing SFE with GSH, we aimed to improve the quality of frozen-thawed bull sperm, generally reduced during the freezing and thawing processes, and also to decrease the detrimental effects caused by ROS. Using semen from four bulls, we investigated whether GSH supplementation at varying concentrations of SFE affected the frozen-thawed sperm quality and subsequent embryo developmental competence after IVF. The results revealed that although sperm motility parameters or DNA integrity were decreased in all bulls at high concentrations of GSH-SFE, *in vitro* embryo development rates and the quality of those embryos were significantly improved in three of the bulls by GSH supplementation.

GSH supplementation has previously been reported to have positive effects on sperm motility in the bull [6, 20, 33] and other species [21, 22, 34], mainly due to a GSH-induced reduction in ROS. In our study, frozen-thawed semen prepared with GSH-SFE did not improve sperm motility in any of the bulls. All bulls showed a tendency for decreased sperm motility at 10 mM GSH-SFE, but this effect was significant for four out of five parameters in three bulls. Comparatively, most bull semen samples showed very little change in motility that agrees with similar reports of no effect of GSH on sperm motility in bulls [18, 19]. Other studies have shown that detrimental effects are occasionally observed under excess concentrations of GSH-SFE in equine [35] and ram [36] semen due to osmotic stress [36]. The deviation from the normal range of osmotic pressure is one of the reasons for motility loss in sperm [37]. We verified that the osmotic pressure applied in this study was within the normal range; therefore, it is unlikely that the motility loss with GSH supplementation in this study was a result of osmotic stress.

Differences in ROS sensitivity among ejaculated semen [38] and dose-dependent changes in antioxidative performance from 0 to 5 mM GSH supplementation have previously been reported [22]. Our results showed no effect of GSH treatment at different concentrations on BAP scores as an indicator of antioxidant power and MDA concentration as an indicator of lipid peroxidation. It was suggested that the GSH concentrations in this study did not affect the antioxidant capacity or cell stress caused by ROS.

Acrosome integrity is another indicator of sperm quality, and bull semen with low acrosome integrity often undergoes premature capacitation *in vitro* [39]. Our results showed no significant differences in sperm viability or acrosome integrity among all bulls at all tested GSH concentrations, agreeing with a previous report on ram semen [36]. Our results suggest that the improved IVF outcome is not necessarily related to semen quality as there was no significant increase in sperm viability or acrosome integrity with GSH-SFE.

In this study, differences in semen quality and IVF results among bulls were observed in fresh and frozen-thawed control semen samples. However, the effect of GSH varied among bulls regardless of the initial conditions. The individual variation in sperm DFI among the four bulls in this study is similar to that reported in a previous study on DFI fluctuation across ten bulls [25]. In Bull 1 and 2, DFI (entirely positive, +) in the 10 mM GSH-SFE group was higher than that of the fresh or 0 mM GSH control groups, and there was a trend of increasing DFI with increasing GSH concentrations. However, sperm viability evaluated using PI staining was not affected by GSH supplementation in SFE. Similarly, a decrease in DNA integrity, evaluated by the comet assay with 5 mM GSH supplementation in the thawing medium was previously reported in boar [40]. In contrast, positive effects of GSH treatment on DNA integrity and embryo production have been shown in Holstein bulls [41] and *in vivo* fertility in boar [42] and buffalo bulls [20]. Fresh semen from subfertile Holstein bulls has also been shown to have a higher DFI (> 20%) than that from the average or high fertility bulls (< 15%) [4]. In our study, none of the bulls had fresh and frozen-thawed control semen with high DFI (entirely positive, +), but two of the four bulls (Bull 2 and 4) showed high DFI (> 20% of entirely positive sperm, +) in frozen-thawed semen prepared with GSH-SFE. Sperm treatment with high concentrations of GSH may lead to increased DFI, especially in bulls that originally tend to have high DFI and might have adverse effects on conception rates. However, this will require further verification.

Although supplementation of GSH in SFE decreased DNA integrity in spermatozoa, it did not hinder fertilization and subsequent *in vitro* embryo development rates. Supplementation with GSH in SFE did not affect sperm penetration or normal fertilization rates. However, pronuclear formation rates at 9 h after IVF, cleavage rates, and embryo development rates increased in frozen-thawed semen prepared with GSH-SFE in three of the four bulls. Despite lower sperm DNA integrity in the two bulls, embryo production after IVF was still high. Interestingly, although the sperm DFI was increased by GSH supplementation, the DFI of embryos was reduced, and the ICM ratio was increased in blastocysts. Higher GSH concentration increased blastocyst formation rates, although these results varied among individual bulls.

Notably, the evaluated DFI was determined from the average value of the total spermatozoa in the sample, indicating that the DNA integrity of the semen sample that actually contributed to fertilization was not profiled. Moreover, spermatozoa categorized as "partially positive" represented fluorescence in the post-acrosomal region. This post-acrosomal region is regarded as the first component of the sperm head to solubilize within the ooplasm [43]. The mechanism of extracellular GSH increasing DFI in sperm, as well as the mechanism of improving embryo development after IVF, remain unknown.

Previous studies have shown that GSH contributes to redox reactions during fertilization and plays an important role in sperm decondensation by decreasing the disulfide bonds in sperm protamine [44, 45]. Sperm pretreatment with GSH before intracytoplasmic sperm injection improves the cleavage rate and quality of embryos and reduces disulfide bonds [33]. Hamilton et al. revealed the functional participation of GSTO2 (Glutathione-S-transferase omega 2) —a major enzyme that facilitates redox reactions through conjugation with GSH— in sperm decondensation and subsequent embryo development [46]. The importance of sperm decondensation in embryo development has also been reported [46]. The inhibition of GSTO2 causes a delay in early sperm nuclear decondensation at 2.5 h after insemination, resulting in decreased blastocyst formation rates [46]. In this study, male PN formation rates at 9 h after IVF were increased in the GSH-SFE groups. However, no differences were observed after 18 h. The earlier effects of GSH may be related to male PN formation that could be overlooked as a normal two-PN condition at 18 h. Moreover, GSH-SFE might play a role as a disulfide-reducing agent for sperm DNA that could be advantageous for early embryonic development stages, as previously reported [33]. Additional investigation is required regarding the increase in sperm DFI caused by GSH supplementation.

Our results revealed a variation in the effect of GSH-SFE on IVF embryo development between individual bulls. Two of the bulls showed both improved cleavage and blastocyst formation rate, while one other showed only an improved cleavage rate. Mammalian semen contains endogenous GSH, although the GSH concentration varies among individuals due to external and other factors [17, 47].

Intracellular GSH levels in bull semen vary in the range of 247–776 pmol/mg and decrease to 44–332 pmol/mg during cryopreservation with EYT extender [5, 15]. This large variation could be responsible for differences in the efficacy of supplemented GSH-SFE in individual bulls. It remains unclear whether it is possible to achieve both the general beneficial effects of GSH and those considered in this study, namely antioxidant activity, improved embryo development, and decreased DNA damage.

In conclusion, GSH supplementation in SFE has the potential to improve embryo development after IVF with frozen-thawed bull semen. However, the effective concentration of GSH supplementation in SFE may vary depending on the individual bull. Further studies are needed to clarify the mechanism of action of GSH treatment, as well as its advantages for calf production in bovine-assisted reproduction.

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

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