

Effects of disulfide bond reducing agents on sperm chromatin structural integrity and developmental competence of *in vitro* matured oocytes after intracytoplasmic sperm injection in pigs

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Abstract

We recently reported that electrical activation followed by secondary chemical activation greatly enhanced the developmental competence of *in vitro* matured porcine oocytes fertilized by intracytoplasmic sperm injection (ICSI). We hypothesized that sperm treatment with disulfide bond reducing agents will enhance the development competence of porcine embryos produced by this ICSI procedure. We examined the effects of glutathione (GSH), dithiothreitol (DTT), GSH or DTT in combination with heparin on sperm DNA structure, paternal chromosomal integrity, pronuclear formation, and developmental competence of *in vitro* matured porcine oocytes after ICSI. Acridine orange staining and flow cytometry based sperm chromatin structure assay were used to determine sperm DNA integrity by calculating the cells outside the main population (COMP α T). No differences were observed in COMP α T values among GSH-treated and control groups. COMP α T values in GSH-treated groups were significantly lower than that in DTT-treated groups. Following ICSI, GSH treatments did not significantly alter paternal chromosomal integrity. Paternal chromosomal integrity in sperm treated with DTT plus or minus heparin was also the lowest among all groups. GSH-treated sperm yielded the highest rates of normal fertilization and blastocyst formation, which were significantly higher than that of control and DTT-treated groups. The majority of blastocysts derived from control and GSH-treated spermatozoa were diploid, whereas blastocysts derived from DTT-treated spermatozoa were haploid. In conclusion, sperm treatment with GSH enhanced the developmental capacity of porcine embryos produced by our optimized ICSI procedure.

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Introduction

Intracytoplasmic sperm injection (ICSI) describes a procedure in which a single sperm is injected directly into the cytoplasm of an oocyte to facilitate fertilization. Clinically, this assisted fertilization approach is presently used for treating male infertility in men with an extremely low number of and/or immotile spermatozoa (Tesarik 1996). ICSI is also a powerful means of producing transgenic animals by sperm-mediated gene transfer (Perry *et al.* 1999). In comparison to the commonly used pronuclear microinjection procedure, ICSI offers higher integration frequency of exogenous genes into the genome of the founder offsprings. Moreover, ICSI makes it possible to introduce large DNA molecules such as yeast or mammalian artificial chromosomes (>250

kilobases in size) into an oocyte without the threat of 'shearing' of the DNA molecule (Perry *et al.* 1999, Martin 2000). Thus, ICSI is considered to be a very useful technique for generating transgenic pigs (Lai *et al.* 2001).

The application of ICSI in pigs has not been as simple as originally thought (Nakai *et al.* 2003). It has been reported that fertilization rate by ICSI is low in pigs, e.g., ~20% on average (Kolbe & Holtz 2000, Martin 2000, Nakai *et al.* 2003, Watanabe & Fukui 2006, Katayama *et al.* 2007). Although the causes of the low success rate in porcine ICSI are uncertain, incomplete oocyte activation (Kolbe & Holtz 1999, 2000) and failure of male pronucleus formation have been suspected as the two pivotal factors for causing the low developmental capacity of ICSI-fertilized porcine embryos

(Thibault 1972, Kren *et al.* 2003, Lee *et al.* 2003). More recently, we reported that electrical activation followed by a secondary chemical activation with 6-dimethylaminopurine (6-DMAP) could significantly enhance the activation rate of porcine oocytes (Tian *et al.* 2006). However, the sperm treatment regimen for maximizing the developmental capacity of ICSI-fertilized porcine embryos has yet to be determined.

During normal fertilization, *in vivo* sperm nuclear decondensation requires two major steps, i.e., reduction of S–S to S–H in protamines followed by replacement of protamines with histones (Zirkin *et al.* 1989). Reduction of S–S to S–H depends on the presence of the free thiol glutathione (GSH; Perreault *et al.* 1988, Zirkin *et al.* 1989). Treatment with dithiothreitol (DTT) to reduce disulfide bonds in protamines, prior to ICSI, enhanced spermatozoa chromosomal decondensation and subsequent formation of a male pronucleus in bovine zygotes (Rho *et al.* 1998). However, DTT-treated sperm did not improve the developmental competence of porcine embryos (Yong *et al.* 2005, Nakai *et al.* 2006). By contrast, when combined with detergents, DTT was detrimental to embryonic development as it breaks paternal chromosomes in the zygote (Szczygiel & Ward 2002). Thus, new methods for sperm preparation are needed for improving the developmental competence of ICSI-fertilized porcine embryos. As an endogenous disulfide bond reducer, reduced GSH is present in the cytoplasm of oocytes, which could protect oocytes against oxidative stress (Perreault *et al.* 1984, 1988). This knowledge has directed the use of GSH in inducing *in vitro* sperm decondensation in human (Reyes *et al.* 2005), bovine (Delgado *et al.* 2001), and hamster zygotes (Francisco *et al.* 1998). To the best of our knowledge, GSH has not yet been tested for sperm preparation to improve the developmental competence of ICSI-fertilized porcine embryos.

Previous studies have shown that heparin can decondense sperm nuclei *in vivo* (Lalich *et al.* 1989, Montag *et al.* 1992). Heparin and other polyanions induce sperm nuclear decondensation by depletion of protamines from the chromatin (Jager *et al.* 1990). In the presence of a disulphide bond reducing agent, relatively low concentrations of heparin induced rapid nuclear decondensation in bovine, mouse, and human sperm (Jager *et al.* 1990). Other polyanions also can decondense sperm nuclei, but with lesser efficacy than heparin (Jager *et al.* 1990). Romanato *et al.* (2003) reported that the ability of heparin to decondense human sperm *in vitro* is related to its structural characteristics rather than its nature of being a polyanion. Whether heparin in combination with other disulphide bond reducing agents (i.e., GSH, DTT) enhances porcine sperm nuclear decondensation is presently unknown. Based on an oocyte activation protocol optimized in our laboratory (Tian *et al.* 2006), the present series of experiments were performed to study: 1) the effects of GSH, DTT, GSH or

DTT in combination with heparin on porcine sperm chromatin structure; 2) the effects of these sperm treatments on the paternal chromatin structure; and 3) the developmental competence of the porcine ICSI-fertilized embryos using these treated sperm *in vitro*.

Results

Effects of disulfide reducing chemical treatments on sperm chromatin structure

After treatment with different disulfide reducing chemicals, spermatozoa were stained with AO and evaluated for chromatin structure by flow cytometry. The results were expressed as the mean (\bar{X} α T) and SD (SD α T) of α T distribution, the percentage of cells with high α T values referred to as cells outside the main population (COMP α T), and the mean (\bar{X} Green) and SD (SD Green) of the green fluorescence intensity distribution. The results are summarized in Fig. 1 and Table 1.

As listed in Table 1, the mean values of α T were significantly different among all groups ($P < 0.05$). The α T values of treatments with DTT in the absence (597.7 ± 7.7) or presence (578.3 ± 9.0) of heparin significantly increased compared with those of control (250.9 ± 2.3) and treatments with GSH in the absence (229.1 ± 5.4) and presence (263.2 ± 4.4) of heparin. No differences ($P > 0.05$) were observed in the COMP α T values among control and GSH treatments with or without heparin. The values of COMP α T markedly increased as mean values of α T increased. The values of COMP α T in DTT treatments without (29.5 ± 0.6) and with (36.2 ± 10.9) heparin were higher ($P < 0.05$) than those in untreated control (3.7 ± 0.6) and GSH without (7.0 ± 0.8) or with (12.2 ± 2.4) heparin. As the COMP α T value increases with sperm chromatin damage, DTT treatments apparently caused a significant increase in the sperm DNA damage. The mean values of the green fluorescence intensity distributions were lower ($P < 0.05$) in control (481.7 ± 25.5), GSH (470.8 ± 5.0), and GSH + heparin (537.8 ± 5.4) groups than those in DTT (645.9 ± 8.5) and DTT + heparin (849.8 ± 78.1) groups. No significant differences were observed in the mean values of the green fluorescence intensity distribution among control and GSH without or with heparin treatments ($P < 0.05$).

Chromosomal integrity in ICSI-fertilized zygotes

The chromosomal integrity of the ICSI-fertilized zygotes before the first cleavage was examined and data are summarized in Table 2. Treatment with DTT minus (8.92%) or plus (5%) heparin did not cause significant increases in the chromosomal damage of the paternal chromosomes with minor or multiple aberrations compared with that of control (1.72%) and treatments with GSH in the absence (1.75%) and presence (5%) of heparin ($P > 0.05$).

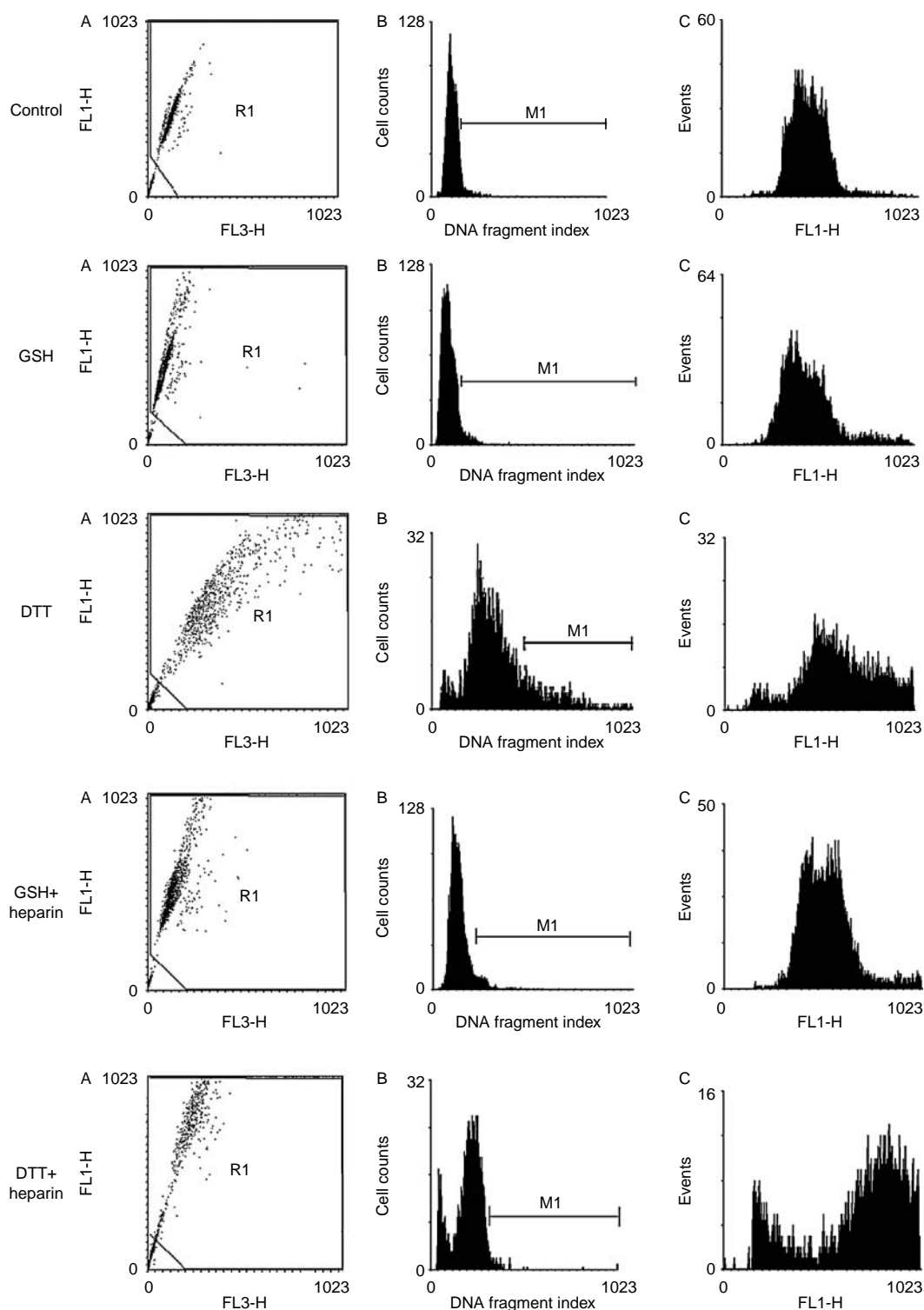


Figure 1 Typical histogram of the AO fluorescence patterns from porcine spermatozoa treated with different disulfide bond reducing agents. A total of 10 000 events were measured and accumulated in the cytogram, each dot representing one spermatozoon. (A) Representative green (FL1-H) versus red (FL3-H) fluorescence bivariate histogram; (B) DNA fragment index (ratio of red to red+green fluorescence) intensity; (C) green fluorescence distributions.

Table 1 Effects of different disulfide bond reducing agents on porcine sperm chromatin structure assay by flow cytometry.

FCM SCSA	Control	GSH	DTT	GSH+heparin	DTT+heparin
X α T	250.9 \pm 2.3 [†]	229.1 \pm 5.4*	597.7 \pm 7.7	263.2 \pm 4.4 [‡]	578.3 \pm 9.0 [§]
SD α T	84.3 \pm 11.0*	85.1 \pm 9.3*	176.5 \pm 2.4 [†]	88.2 \pm 8.7*	180.1 \pm 7.0 [†]
COMP α T	3.7 \pm 0.6*	7.0 \pm 0.8*	29.5 \pm 0.6 [†]	12.2 \pm 2.4*	36.2 \pm 10.9 [†]
X Green	481.7 \pm 25.5*	470.8 \pm 5.0*	645.9 \pm 8.5 [†]	537.8 \pm 5.4*	849.8 \pm 78.1 [†]
SD Green	108.8 \pm 14.4*	143.5 \pm 6.1 [†]	198.7 \pm 4.3 [‡]	133.3 \pm 5.0 [†]	194.8 \pm 12.1 [‡]

FCM, flow cytometric method; SCSA, sperm chromatin structure assay; α T, ratio of red to total (red plus green) fluorescence intensity; X α T, the mean value of α T distribution; SD α T, the SD of α T distribution; COMP α T, cells outside the main population; X Green, the mean value of the green fluorescence intensity; SD Green, the SD of the green fluorescence intensity. Values in the same row with different superscripts differ significantly ($P<0.05$).

Effects of sperm treatments with disulfide bond reducing chemicals on pronuclear formation and developmental competence of ICSI-fertilized oocytes

As summarized in Table 3, there were statistical differences ($P<0.05$) among the activation rates of ICSI-fertilized oocytes by using sperm treated with different chemicals. Interestingly, normal fertilization rates of oocytes fertilized by ICSI with GSH (62%), GSH+heparin (51.16%), and DTT+heparin- (45.83%) treated sperm were significantly greater ($P<0.05$) than those of the control (33.33%) and DTT-treated (38.78%) groups. There were no differences among GSH, GSH+heparin, and DTT+heparin-treated groups and between control and DTT-treated groups ($P>0.05$).

The developmental competence of the ICSI-fertilized embryos derived from sperm treated with different disulfide bond reducing reagents is summarized in Table 4. The cleavage rates did not differ among all groups ($P>0.05$); however, the blastocyst formation rates were significantly different among groups ($P<0.05$). GSH-treated sperm yielded the highest of blastocyst formation rate (40.21%), which was significantly higher than that of control (19.60%), DTT- (24.72%), and DTT+heparin- (28%) treated groups ($P<0.05$). The blastocyst formation rate in the GSH+heparin-treated group was also higher than that of control group (32.26% vs 19.60%, $P<0.05$). Sperm treated with DTT in the absence or presence of heparin did not cause significant changes in blastocyst formation rates in comparison with control ($P>0.05$). In addition, the cell number (54.05 ± 4.37) of the blastocyst formed in the DTT+heparin group was

higher ($P<0.05$) than that in control (44.71 ± 6.25), GSH (38.96 ± 4.72), DTT (42.32 ± 4.59), and GSH+heparin (38.92 ± 7.82) groups.

Finally, we examined the karyotype of ICSI blastocysts derived from sperm treated with different chemicals. As summarized in Table 5, there was a dramatically increased haploid blastocyst rate (80%) in the DTT-treated group than in other treatment groups, in which $\sim 60\%$ of the blastocysts formed were diploid ($P<0.05$). No polyploid blastocysts were observed in any of the groups.

Discussion

In most mammals, sperm nuclear decondensation *in vivo* seems to be composed of two distinct stages: reduction of disulfide bonds in protamines and replacement of protamines with histones (Zirkin *et al.* 1989). Therefore, *in vitro*, spermatozoa are often treated with disulfide bond reducing agents such as DTT or GSH for facilitating its nuclear decondensation. DTT is a commonly used disulfide bond reducing reagent, and sperm treatment with DTT has been used to increase the success rate of bovine and porcine ICSI.

In this study, we have first investigated the effects of different disulfide bond reducing agents (DTT, GSH, and their combination) on porcine sperm DNA structure, parental chromosomal integrity, and developmental competence of porcine ICSI embryos derived from the treated sperm. The flow cytometry based sperm chromatin structure assay was used to measure sperm chromatin integrity. This assay exploits the metachromatic properties of acridine orange (AO) to monitor the

Table 2 Chromosomal aberrations in porcine zygotes after injection with spermatozoon treated with different disulfide bond reducing chemicals.

Treatment	No. of metaphases examined	No. of normal metaphases (%)	No. of metaphases with minor aberrations (%)	No. of metaphases with multiple aberrations (%)	Total no. of metaphases with aberrations (%)
Control	58	57 (98.28)*	0 (0)*	1 (1.72)*	1 (1.72)*
GSH	57	55 (96.49)*	0 (0)*	1 (1.75)*	1 (1.75)*
DTT	56	51 (91.07)*	3 (5.36)*	2 (3.57)*	5 (8.92)*
GSH+heparin	56	54 (96.43)*	2 (3.57)*	0 (0)*	2 (3.57)*
DTT+heparin	60	57 (95)*	2 (3.33)*	1 (1.67)*	3 (5)*

Values within a column with different superscripts differ significantly ($P<0.05$).

Table 3 Effects of different disulfide bond reducing chemicals for porcine spermatozoa on pronuclear formation of porcine intracytoplasmic sperm injection embryos.

Nucleus morphology	Treatment				
	Control	GSH	DTT	GSH + heparin	DTT + heparin
No. of oocytes examined	48	50	49	43	48
Total no.(%) oocytes at M α stage	12 (25)*	6 (12)*	12 (24.49)*	8 (18.60)*	5 (10.42)*
No.(%) oocytes with 1MPN+1PB	8 (16.67)*	6 (12)*, [†]	10 (20.41)*	6 (13.95)*, [†]	2 (4.17) [†]
No.(%) oocytes at M α stage+1PB	4 (8.33)*	0 (0)*	2 (4.08)*	2 (4.65)*	3 (6.25)*
Total no.(%) oocytes activated	36 (75)*	44 (88)*	37 (75.51)*	35 (81.40)*	43 (89.58)*
No.(%) oocytes with 1MPN+1FPN+2PB	16 (33.33)*	31 (62) [†]	19 (38.78)*	22 (51.16)*, [†]	22 (45.83)*, [†]
No.(%) oocytes with 1MPN+2FPN+1PB	0 (0)*	0 (0)*	1 (2.04)*	0 (0)*	0 (0)*
No.(%) oocytes with 2FPN+1PB+sperm	4 (8.33)*	0 (0)*	4 (8.16)*	3 (6.98)*	2 (4.17)*
No.(%) oocytes with 1FPN+1PB(or 2PB)+sperm	16 (33.33)*	13 (26)*	13 (26.53)*	10 (23.26)*	19 (39.58)*

Values in within a row with different superscripts are significantly different ($P < 0.05$). FPN, female pronuclear; PB, second polar body; MPN, male pronuclear; sperm, intact or condensed sperm head.

susceptibility of sperm chromatin DNA to acid-induced denaturation *in situ* (Evenson *et al.* 1980, 1991, Spanò & Evenson 1993). The bivariate data can be conveniently expressed as function αT (αT is the ratio of red to total (red plus green) fluorescence intensity (Darzynkiewicz *et al.* 1975), which represents the amount of the denatured, single-stranded DNA over the total cellular DNA. Therefore, αT parameters can reflect the chromatin alterations associated with DNA nicks. When αT value increases, the denatured or single strand DNA binding sites increases and so does the percentage of COMP αT and the percentage of sperm with denatured DNA or damaged chromatin. DTT acts on disulfide bonds of sperm protamine and induces sperm chromatin decondensation (Rho *et al.* 1998, Tatenò & Kamiguchi 1999, Szczygiel & Ward 2002, Yong *et al.* 2005). We found that the value of COMP αT in DTT-treated sperm was higher than those of GSH-treated and control groups. These results indicate that DTT may increase the susceptibility of the sperm's DNA to other insults.

DTT treatment alone did not disrupt the integrity of paternal DNA (Ward *et al.* 2000). However, Szczygiel & Ward (2002) reported that a combination of DTT and detergent causes paternal chromosomal damage in mice. Our current data suggest that without detergent, DTT alone caused more sperm DNA denaturation than untreated control in pigs. The disulfide bonds (–SS–) in proteins make spermatozoa resistant to harsh physical treatments such as freeze-drying. A lack of the extensive –SS– cross-linking makes the nucleus of immature

spermatozoa vulnerable to physical and chemical disruptions (Kaneko *et al.* 2003). DTT, which reduces S–S bonds in the cysteine-rich protamines to –SH, made mouse epididymal spermatozoa susceptible to damage from freeze-drying (Kaneko *et al.* 2003). Protamines are most likely to be responsible for the genetic (chromosomal) integrity of the sperm nucleus in the face of an artificial insult such as freeze-drying (Kaneko *et al.* 2003). Even very subtle alterations in the structural proteins of the nucleus are sufficient to deprive the sperm's DNA of the ability to support embryonic development (Ward *et al.* 2000). Sperm chromatin integrity is of critical importance during ICSI. The endogenous nucleases in mouse sperm can be activated by freeze-thawing, which in turn can cleave DNA in spermatozoa (Pérez-Crespo *et al.* 2008). The uterine content from females mated to vasectomized males and the seminal vesicle fluid have increased nuclease activities. When the epididymal sperm were exposed to these conditions, an increased incidence of sperm chromosome nicks and extremely severe DNA breakages were observed after ICSI. However, chromosomes were normal in sperm exposed to the uterine content from unmated females in estrus (Yamauchi *et al.* 2007). Based on these recent findings, we speculated that the harmful effects of DTT on porcine sperm observed in our present study very likely originated from the effects of sperm endonucleases. It is also possible that DTT destabilized the extensive –SS– cross-linking and altered the structural proteins of the nucleus

Table 4 Developmental competence of *in vitro* matured porcine oocytes fertilized by intracytoplasmic sperm injection using a spermatozoon treated with different disulfide bond reducing chemicals *in vitro*.

Treatment	Oocytes	Number of cleavages (%)	Number of blastocysts (%)	Cell number of blastocysts
Control	71	60 (84.31)*	14 (19.60) [‡]	44.71 \pm 6.25 [†]
GSH	72	63 (87.60)*	29 (40.21)*	38.96 \pm 4.72 [†]
DTT	89	82 (92.13)*	22 (24.72) ^{†,‡}	42.32 \pm 4.59 [†]
GSH + heparin	105	98 (92.86)*	34 (32.26)*, [†]	38.92 \pm 7.82 [†]
DTT + heparin	75	70 (93.33)*	21 (28) ^{†,‡}	54.05 \pm 4.37*

Values within a column with different superscripts differ significantly ($P < 0.05$).

Table 5 Karyotyping of porcine blastocysts derived from *in vitro* matured oocytes fertilized by intracytoplasmic sperm injection with spermatozoon treated with different disulfide bond reducing chemicals.

Treatment	Blastocysts (No.)	Number of haploid (%)	Number of diploid (%)	Number of polyploid (%)
Control	8	3 (37.5)*	5 (62.5)*	0
GSH	9	3 (33.33)*	6 (66.67)*	0
DTT	10	8 (80) [†]	2 (20) [†]	0
GSH+	6	2 (33.33)*	4 (66.67)*	0
heparin				
DTT+	10	4 (40)*	6 (60)*	0
heparin				

Values within a column with different superscripts differ significantly ($P < 0.05$).

in sperm, which makes them more susceptible to nuclease-dependent DNA damage (Sutovsky *et al.* 1997, Ward & Ward 2004).

In the present study, we also used another disulfide bond reducer, GSH, to treat porcine spermatozoa. GSH, the most abundant low-molecular-weight non-protein thiol, exists in two forms: reduced GSH and GSH disulfide (GSSG). It has been clearly shown that GSH is a disulfide bond reducer present in the cytoplasm of oocytes (Perreault *et al.* 1984, 1988). Oocytes can synthesize GSH (tripeptide γ -glutamyl-cysteinyl-glycine; GSH) during the first meiosis. Oocyte-derived GSH seems to assure the reduction of disulfide bonds in sperm nucleus. This, in turn, promotes nuclear decondensation for male pronucleus formation during fertilization (reviewed by Sutovsky & Schatten 1997). Thus, GSH provides the reducing power to initiate chromatin decondensation, prior to the male pronucleus formation (Yoshida *et al.* 1993, Funahashi *et al.* 1994). Depletion of endogenous GSH by a specific inhibitor of GSH synthesis during bovine oocyte maturation blocks the formation of a male pronucleus and prevents the assembly of sperm aster microtubules (Sutovsky & Schatten 1997). The elevated levels of oocyte GSH can enhance male pronuclear formation after IVF (Funahashi *et al.* 1994). In this study, we found that in comparison to DTT, GSH treatments resulted in much lower values of α T and COMP α T in porcine sperm, indicating that GSH treatment does not cause significant DNA denaturation. These results suggested that GSH may function as a disulfide bond reducer with fewer side-effects, thus rendering it more suitable for promoting sperm chromatin decondensation *in vitro*. In addition, GSH is a potent antioxidant that can counteract the effects of oxidative stress induced sperm DNA damage due to increased reactive oxygen species production and subsequent plasmalemma lipid peroxidation, intracellular enzyme leakage, and eventually chromatin damage (Aitken 1999, Lubarda 2001). Thus, the antioxidant property of GSH may also play a role in protecting porcine sperm

DNA against DTT-induced damage as revealed in our present study.

Furthermore, chromosomal aberrations in ICSI-fertilized embryos were examined to validate the effects of different disulfide bond reducing chemicals on sperm chromatin integrity. We found that treatments with DTT caused more paternal DNA damage with minor or multiple aberrations in porcine sperm than that of GSH-treated and control groups, although the difference did not reach statistical significance. These data were consistent with the results of sperm chromatin structure assay (SCSA). Moreover, these observations favor the idea that in comparison with DTT, GSH is a more suitable agent for promoting porcine sperm chromatin decondensation in porcine ICSI.

We also examined the effects of different disulfide bond reducing chemicals on pronuclear formation in porcine zygotes and the *in vitro* developmental competence of porcine oocytes fertilized with the treated sperm by ICSI. In comparison with DTT, treatment with GSH resulted in significantly higher rates of zygotes with pronucleus and blastocyst formed. It has been shown that pretreatment of spermatozoa with DTT could enhance the overall success rate of ICSI-fertilized embryos in cows (Rho *et al.* 1998, Suttern *et al.* 2000) and minke whales (Asada *et al.* 2001). By contrast to these previous reports, we did not observe a tendency of increased rate of blastocyst formation in porcine embryos fertilized by ICSI with DTT-treated sperm. However, our data agreed with those of Nakai *et al.* (2006). The rates of pronucleus and blastocyst formation *in vitro* could not be improved by spermatozoa treatment with DTT. Ward *et al.* (2000) found that DTT treatment destabilized sperm nuclear matrix in the presence of an ionic detergent and oocytes injected with these nuclei failed to develop. The discrepancies among these studies may be explained by the fact that although treatment with DTT could reduce S-S in the sperm protamines, simultaneous paternal DNA damage caused by DTT would affect subsequent development of zygotes. In our present study, DTT-treated sperm did not increase the rate of pronuclear and blastocyst formation in comparison with GSH. Intriguingly, we observed a higher haploid rate (80%) of the karyotype of ICSI-fertilized blastocysts derived from DTT-treated sperm. The following may offer some hints for the causes of this phenomenon. First, in the majority of ICSI zygotes, the second polar body was extruded by our optimized oocyte activation protocol (Tian *et al.* 2006) and this may have rendered the zygotes with a haploid suit of maternal chromatin. Alternatively, DTT may elicit harmful effects on porcine sperm chromatin, which in turn causes negative effects on the development of male pronucleus in ICSI-fertilized porcine embryos. Thus, we speculated that the chromatin in haploid ICSI blastocyst was only originated from the maternal chromatin of DTT group. Moreover, our results were similar to those

reported in humans (Aoki *et al.* 2005). In human ICSI, it has been shown that the sperm DNA damage is detrimental to fertility and has been regarded as a major factor in lowering embryo quality (Tomsu *et al.* 2002, Virant-Klun *et al.* 2002), blastulation rate (Seli *et al.* 2004), and overall pregnancy rate after IVF (Evenson *et al.* 2002, Bungum *et al.* 2004, Henkel *et al.* 2004, Virro *et al.* 2004).

Heparin was also used to induce nuclear decondensation (Vendrell *et al.* 1998, Delgado *et al.* 2001, Romanato *et al.* 2003). The glycoprotein heparin receptors are present on human sperm plasma membrane (Delgado *et al.* 1982). Binding of heparin to its receptors destabilizes sperm plasma membrane that in turn facilitates the incorporation of other molecules such as GSH into sperm nucleus (Marina *et al.* 2003). Furthermore, the strong binding affinity of heparin for protamines can assist the formation of a highly insoluble complex that may promote sperm chromatin decondensation (reviewed in Romanato *et al.* 2003). Combination of heparin and DTT may help to induce swelling of sperm nuclear matrix (Jager *et al.* 1990). These combined actions of both compounds loosen DNA-protamine complexes, which causes a progressive decondensation of nuclear matrix (Leopoldo *et al.* 2004). Alternatively, the interactions between heparin and GSH may induce sperm nuclear decondensation via an ionic chromatin charge neutralization mechanism (Delgado *et al.* 2001) in bovine (Delgado *et al.* 2001), hamster (Francisco *et al.* 1998), and human (Reyes *et al.* 2005) zygotes. However, another study found that heparin alone induces sperm nuclear decondensation when only the membranes are severely damaged either by freezing and thawing or by detergents (Jager *et al.* 1990). In this study, we found that the values of COMP α T and the parental chromosomal aberrations of DTT-treated porcine sperm did not differ significantly from that of DTT+heparin-treated porcine sperm nor between GSH and GSH+heparin-treated porcine sperm. Moreover, DTT combined with heparin did not improve the developmental competence of porcine ICSI-fertilized embryos. By contrast, the fertilization rates and developmental competence of porcine ICSI-fertilized embryos derived from GSH-treated spermatozoa were higher than in those derived from GSH+heparin-treated spermatozoa. Why heparin has no detectable effects on porcine sperm chromatin decondensation is not clear. We speculate that the sperm used in our study might be the reason. At the time of freezing, the sperm used were diluted in media with a cryoprotectant. Prior to experimental use, the sperm were only subjected to one cycle of freezing/thawing. Thus, the plasma membrane of the spermatozoa was not destroyed and heparin was unable to enter sperm cells to elicit its effect when used alone. However, we do not know why heparin applied with GSH or DTT did not have synergic effects to induce porcine sperm nuclear decondensation. Whether species differences contribute

to the discrepancies of these studies awaits further investigation.

In conclusion, based on our specific oocyte activation protocol, we found that the overall efficiency of porcine ICSI-fertilized embryos was significantly increased when spermatozoa were treated with GSH. Compared with DTT, GSH could decrease DNA breakage in spermatozoa, maintain paternal chromosome integrity, enhance pronuclear formation, and developmental competence of ICSI-fertilized porcine embryos *in vitro*. Thus, in comparison with the commonly used DTT procedure, our present study demonstrated that GSH is a more suitable disulfide bond reducing agent for preparing porcine sperm for ICSI.

Materials and Methods

Chemicals and reagents

All chemicals and reagents, unless otherwise stated, were purchased from Sigma-Aldrich Co. Porcine follicular fluid (pFF) was aspirated from 3–6 mm diameter follicles from ovaries collected at a local slaughterhouse (Beijing Jingbei Slaughterhouse, Beijing, China). The follicular fluids were collected under sterile conditions and stored at -20°C . Prior to use, the follicular fluids were centrifuged at 1500 *g* for 30 min (4°C) as described previously (Abeydeera *et al.* 1998).

Sperm preparation

Fresh semen was diluted 1:10 with the TNE buffer (0.01 M Tris, 0.15 M NaCl, 1 mM EDTA, pH 7.4) supplemented with 10% (v/v) glycerol, and then frozen and stored in liquid nitrogen until in use. Frozen semen samples as described above were thawed at 37°C . Spermatozoa were collected by centrifugation (600 *g*, 6 min) and resuspended in TNE buffer at a concentration of $1 \times 10^6/\text{ml}$. The sperm solution was allocated for treatment with either GSH (8 mM, 120 min), DTT (5 mM, 60 min), GSH (8 mM), and heparin (10 $\mu\text{g}/\text{ml}$) for 60 min, or DTT (5 mM) and heparin (10 $\mu\text{g}/\text{ml}$) for 60 min at 39°C . Sperm without any chemical pretreatment served as the control group. After incubation, the sperm samples were washed three times with TNE buffer by centrifugation (600 *g*, 6 min) to remove the chemicals. After the last wash, the sperm pellet was resuspended in TNE buffer and kept on ice until use.

In this study, the treatment time and concentrations of DTT and heparin were chosen based on previous literature (Rho *et al.* 1998, Delgado *et al.* 2001) and the treatment time and concentrations of GSH were selected from our pre-experiments (data not shown) by the higher rate of sperm nuclear swelling under phase contrast light microscopy.

AO staining and flow cytometry

The chromatin integrity of spermatozoa was examined by AO staining and flow cytometry as previously described (Evenson *et al.* 1986, Evenson 1990). Semen samples (200 μl) were treated with 400 μl of a low pH solution with detergent (0.1%

Triton X-100 (v/v), 0.15 M NaCl and 0.08 M HCl, pH 1.2) for 30 s. Triton X-100 treatment permeabilizes sperm cell membranes and thus provides greater accessibility of AO to stain DNA. After the 30 s acid treatment, 1.2 ml AO staining buffer (6 µg/ml AO, 0.1 M citric acid, 0.2 M Na₂HPO₄, 1 mM EDTA, 0.15 M NaCl, pH 6.0) were added to stain DNA for 3 min. The spermatozoa were then analyzed by a FACS Calibur flow cytometer (Becton Dickinson, San José, CA, USA), equipped with a 6 W Argon ion laser (Innova 306, Coherent, Santa Clara, CA, USA), tuned at 488 nm and operated at a power output of 300 mW, light mode. Green (530 ± 30 nm) and red (> 630 nm) fluorescence intensities, corresponding to amounts of native and denatured DNA respectively, were recorded from 1 × 10⁴ spermatozoa per sample. All measurements were initiated at 3 min after AO staining with a flow rate of ~200 cells/s. Scattergram analysis was carried out using the Cell Quest Pro version 4.0 software (Becton Dickinson Biosciences). Each data point represents the coordinate of red and green fluorescence intensities for an individual spermatozoon. Events accumulated in the lower left corner correspond to cell debris and were excluded from the analysis (Fig. 1). The bivariate data can be conveniently expressed by the function αT , which is the ratio of red to total (red plus green) fluorescence intensity (Darzynkiewicz *et al.* 1975), thus representing the amount of denatured, single-stranded DNA over total cellular DNA. αT was calculated (ListView; Phoenix Flow Systems, San Diego, CA, USA) for each sample. The results were expressed as the mean ($\bar{X} \alpha T$) and the SD ($SD \alpha T$) of αT distribution, and the frequency of cells with high αT values referred as to COMP αT . The values of αT ranged between 0 and 1, but were converted to 0–1000 numerical scale to facilitate working with integral numbers. Additional parameter in this flow cytometry based sperm chromatin structure assay included the mean (\bar{X} Green) of the green fluorescence intensity.

Oocyte collection and in vitro maturation (IVM)

Porcine ovaries were obtained from gilts at a local abattoir and transported to the laboratory in sterile physiological saline (Beijing Chemical Reagent Co., Beijing, China) containing 75 mg/l penicillin (Huabei Medicine Co. Ltd, Shijiazhuang, China) and 50 mg/l streptomycin (Hebei Yuanzheng Medicine Co. Ltd, Shijiazhuang, China) at 30 °C to 35 °C within 2 h. The ovaries were washed three times with fresh saline and stored in saline at 37 °C. The cumulus-oocyte complexes (COCs) were aspirated from the antral follicles (3–6 mm in diameter) using a 16-gauge needle attached to a 10 ml disposable syringe. The aspirates were transferred into a 15 ml plastic centrifuge tube pre-warmed to 37 °C in a water bath. The sediments were washed twice with HEPES-buffered Tyrode albumin lactate pyruvate medium (TL-HEPES; Bavister *et al.* 1983) containing 0.3% (w/v) BSA (Roche Diagnostics Corporation). Only those oocytes surrounded by at least three layers of compact cumulus cells and with uniform cytoplasm were selected for experimental use. The COCs were washed three times with fresh maturation medium composed of TCM199 medium (Earle's Salts; Gibco, Life Technologies Inc.; Abeydeera *et al.* 2000, Hyun *et al.* 2003) supplemented with 10% (v/v) PFF, 0.1 mg/ml

cysteine, 10 ng/ml epidermal growth factor, 10 IU/ml eCG (Intervet Pty. Ltd, Boxmeer, Australia), 10 IU/ml hCG (Intervet Pty. Ltd), 0.065 mg/ml penicillin and 0.05 mg/ml streptomycin. Selected COCs (25–30/group) were transferred to a droplet of 100 µl maturation medium covered with mineral oil in a petri dish (Nunc, Nalge Nunc International, Roskilde, Denmark). IVM was carried out at 39 °C in an atmosphere of 5% CO₂ in humidity air for 44 h as described previously (Abeydeera *et al.* 2001).

ICSI

After IVM, COCs with expanded layers of cumulus cells were treated with 0.1% (w/v) hyaluronidase. Cumulus cells were then removed by repeated gentle pipetting. Denuded oocytes were placed in droplets of TCM199-HEPES containing 0.5% (v/v) fetal bovine serum (FBS; Hyclone, Logan, Utah, USA). Denuded oocytes with uniform cytoplasm were chosen for the following experiments.

ICSI was performed by using an inverted microscope (Olympus IX70, Olympus optical Co. Ltd., Tokyo, Japan) with micromanipulators (Eppendorf, Hamburg, Germany). To accommodate the bigger size of a porcine spermatozoon, the injection pipettes had to be 6–7 µm in their inner diameter. A 100 µl drop of HEPES-M199 containing 0.5% (v/v) FBS and a 20 µl drop of 10% (w/v) polyvinylpyrrolidone (PVP) was placed in the center of a 35 mm dish covered with mineral oil. For each group of injections, 20–30 oocytes were placed in a 100 µl drop of medium and the 20 µl sperm suspension was transferred to the PVP drop that had been pre-equilibrated for 2 h. The oocyte was held with a holding pipette in an orientation so that the first polar body was at the 6 or 12 o'clock position. A spermatozoon was then injected into the cytoplasm with a micropipette.

Activation of oocytes following ICSI

After spermatozoon injection, oocytes were transferred into an electrical activation medium (0.3 M mannitol, 0.05 mM CaCl₂, 0.1 mM MgCl₂; Zimmermann & Viemken 1982). The oocytes were then placed in a chamber connected to an electrical pulse generator (Fujira Industry Co. Ltd, Tokyo, Japan) with two parallel electrodes at a distance of 0.46 mm. Direct current pulse (1.3 kV/cm, 1 × 80 µs) was applied to activate the oocytes (Zhu *et al.* 2002). After electrical activation, the oocytes were washed three times with PZM-3 (Yoshioka *et al.* 2002) medium containing 3 mg/ml BSA and cultured in the same medium for 3 h. The ICSI oocytes were then cultured for another 6 h in droplets of PZM-3 medium containing 3 mg/ml BSA supplemented with 2 mM 6-DMAP for chemical activation.

In vitro embryo culture

The ICSI oocytes were washed three times with PZM-3 medium containing 3 mg/ml BSA. 15–20 oocytes/groups were cultured in a 50 µl droplet of PZM-3 medium droplets containing 3 mg/ml BSA under mineral oil at 39 °C in a humidified air of 5% CO₂ for 7 days. The cleavage and blastocyst rates of the ICSI

oocytes were recorded at day 2 and 7 respectively. Three replicates were performed for this experiment.

Chromosomal analysis

Following ICSI and electrical activation, the zygotes were cultured in droplets of PZM-3 medium containing 3 mg/ml BSA for 6 h and then transferred into PZM-3 medium containing 3 mg/ml BSA supplemented with 0.05 µg/ml of colchicine. Between 19 and 21 h after ICSI, eggs arrested at the metaphase of the first cleavage were treated with 1% pronase (1000 tyrosine units/mg, Sigma) for 5 min at room temperature to soften the zona pellucida. The zygotes were then treated with a hypotonic solution composed of a mixture of 1% sodium citrate and 30% FBS (1:1, v/v) for 5 min at 37 °C or 10 min at room temperature. Chromosomes were spread on slides by the gradual fixation/air-drying method as described (Mikamo & Kamiguchi 1983). Briefly, zygotes with a small amount of hypotonic solution were transferred into fixative I (methanol:glacial acetic acid:H₂O = 5:1:4) for 3–5 min until they turned pale. The zygotes were then aspirated into a fine-mouthed glass pipette and released within a circle that had been made on the reverse side of a grease-free glass slide. The zygotes were covered immediately with fixative II (methanol:glacial acetic acid=3:1) released from another pipette. The slides were kept in a jar filled with fixative II for 5 min. Finally, the slides were placed into fixative II (methanol:glacial acetic acid:H₂O=3:3:1) for 1 min, removed slowly and dried in a stream of warm moist air (Rosenbusch *et al.* 1998). The samples were stained with 10 µg/ml Hoechst 33342 (Sigma) for 10 min and observed at ×1000 magnification with oil-immersion optics using u.v. illumination for conventional chromosome analysis under a microscope (Olympus BX60, Olympus Optical Co. Ltd). A spermatozoon was classified to be chromosomally normal when a zygote contained 38 structurally normal chromosomes (19 pairs) at metaphase of the first mitotic division. We looked at the percentage of normal and abnormal metaphases (differentiating between metaphases with minor and multiple aberrations). When more than nine aberrations per karyoplate were observed, we categorized them as multiple aberrations. The number of aberrations per spermatozoon, which was calculated by dividing the total number of aberrations by the total number of karyoplates examined in one treatment, represented the intensity of the chromosomal damage. Four replicates were performed of this experiment (Szczygiel & Ward 2002).

Fixation and staining of zygotes

At 15 h after ICSI, zygotes cultured in PZM-3 medium were fixed and stained to assess pronuclear formation as follows. The zygotes were mounted on glass slides, fixed in acetic acid:alcohol (1:3, v/v) for 48 h, stained with 1% (w/v) orcein in 45% (v/v) acetic acid in water. Pronuclear formation was examined under a phase contrast microscope (Olympus IX70, Olympus Optical Co. Ltd). Oocytes with one pronucleus and a non-condensing sperm head were classified as activated,

whereas those with two pronuclei and without a non-condensing sperm head were regarded as fertilized (Rho *et al.* 1998, Lee *et al.* 2003).

Chromosome analysis of ICSI blastocysts

Blastocysts were synchronized at metaphase by the addition of 0.5 µg/ml colchicine to the culture medium for 4 h, followed by incubation in 1% (w/v) sodium citrate for 20 min at 4 °C. Each individual blastocyst in 1.5 µl methanol:acetic acid (1:1) was placed on a pre-cleaned microscope slide and blown upon to spread the chromosomes as they dried. The samples were stained in Hoechst 33342 (10 µg/ml) and chromosomes were examined under u.v. illumination under a microscope as described above. Blastocysts were classified as diploid (2n), haploid (1n), and polyploid/mixoploid (≥3n/blastocysts with blastomeres of different ploidy) according to the chromosome numbers.

Statistical analysis

Analysis of variance was carried out by use of ANOVA (PROC GLM) using the SAS statistical package (SAS Institute Inc., Cary, NC, USA). All percentage data were subjected to arcsine transformation before statistical analysis. Duncan's multiple range tests and χ^2 were used to compare values of each individual treatment. $P < 0.05$ was considered to be statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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