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ORIGINAL ARTICLE

Effect of glutathione on the development of rat embryos following microinsemination

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Abstract
Purpose The present study was carried out to evaluate the role of glutathione on rat embryo developmental potential after ICSI. We observed the effects of glutathione on the development of non-treated rat embryos, ICSI embryos and embryos with sham injection treatment. The development of glutathione-microinjected embryos was also observed.
Methods Oocytes and fertilized embryos were obtained from superovulated Wistar–Imamichi rats and cultured in mR1ECM medium. Oocytes and embryos were then allowed to develop to assess the effect of glutathione on the development rate in intact embryos, micro-injected embryos and ICSI embryos.
Results (1) In the intact embryo, the proportion of blastocyst stage development increased when 0.01 mM GSH was added to the medium compared to the control. (2) Microinjection of glutathione (GSSG, GSH) into the embryo increased development at each stage, and the addition of 0.2 nM GSSG or GSH significantly increased blastocyst development, in comparison to that of the control ($P < 0.05$). (3) Compared to the control, all the GSSG and GSH concentrations improved damaged blastocyst development, where 0.01 mM GSH improved significantly ($P < 0.05$). (4) The addition of glutathione in the medium increased the rate of blastocyst development after ICSI. A significantly higher number of TE and total cells were obtained in the micro-injected embryo with both of the 0.02 mM GSSG and GSH treatments ($P < 0.05$).
Conclusions The addition of glutathione into the culture media can improve early embryo development and is capable of repairing the damage of ICSI rat embryos.

Keywords Blastocyst · Glutathione · ICM · ICSI · Rat embryo

Introduction

Intracytoplasmic sperm injection (ICSI) is a technique used to fertilize oocytes by delivering spermatozoa directly into the ooplasm using micromanipulating devices. Mammalian ICSI was initially designed to examine the fertilization steps in the ooplasm after delivering sperm heads from epididymal or testicular spermatozoa [1, 2].

In 1995, Kimura and Yanagimachi [3] established highly reproducible ICSI in mice, confirming that in laboratory species, at least some oocytes fertilized by direct injection with spermatozoa could develop to term. In rats, in general, however, a high degree of skill is needed for successful ICSI because of technical or biological problems, such as fragile oocytes, large sperm heads [4], and arrest of in vitro rats embryo development at the 2-cell and 4-cell stages [5]. There is evidence that glutathione has established function in various reproductive processes. Glutathione is the major non-protein sulphydryl compound in mammalian cells and it is known to protect the cell from oxidative damage [6]. Several studies also suggested that glutathione may play an important role in many biological processes including DNA and protein synthesis, cellular

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protection during oxidative stress, and cell proliferation during embryonic events [7–9]. González-Fernandez et al. [10] found a prominent presence of glutaredoxins in rat oocytes and in corpora lutea during the developmental and oestrous cycle changes. Glutathione was discovered in the reproductive tract fluid of mice and was found to improve development of preimplantation embryos after chemically induced depletion of glutathione [11].

Thus, the objectives of the present study were to investigate the effects of glutathione on the development of non-treated rat embryos, ICSI embryos and embryos with sham injection treatment. The development of glutathione-microinjected embryos was also observed.

Materials and methods

Animals, embryo collection and experimental procedure

The Wistar–Imamichi rats (8–10 week) were used throughout the experiments. They were offered feed with a standard diet and tap water ad libitum. Animals were kept in polycarbonate cage with wood shavings under a 12 h light: 12 h dark regimen (light on at 6:00), at a temperature of 20 ± 1°C in accordance with the "Guideline for Regulation of Animal Experimentation, Faculty of Agriculture, Shinshu University."

Experiment 1: the effect of glutathione on embryo development

Rats were induced to superovulate with injections of 30 IU PMSG (pregnant mare serum gonadotropin; Sankyo, Co., Japan) and 30 IU of hCG (human chorionic gonadotropin; Sankyo, Co., Japan) given 54 h apart. After the superovulation, female rats were mated with fertile males to obtain the fertilized embryos. The presence of a vaginal plug on the following day indicated successful mating, and this was designated as day one of gestation. Oviducts were removed 14–15 h after the injection of hCG and placed in modified rat 1-cell embryo culture medium (mR1ECM) [12] in a Petri dish. Each of 0.01, 0.02 or 0.05 mM glutathione in the form of oxidized (GSSG) and reduced (GSH) (Nacalai Tesque, Kyoto, Japan) was added in the mR1ECM.

Experiment 2: the effect of glutathione micro-injection on embryo development

Fertilized embryos, 12 h after the injection of hCG were collected from dissected fallopian tubes and washed with mR1ECM medium and used immediately for culture or microinjection. GSSG or GSH (1 μl) was injected into the embryos and final concentration was 0.2, 0.5 or 1.0 nM and cultured in the mR1ECM.

Experiment 3: the effect of glutathione on the sham-injected embryo

Fertilized embryos, 12 h after the injection of hCG were sham-injected, except for the absence of sperm in the injected medium and cultured in the mR1ECM. The basic mR1ECM medium was supplemented with 0.01, 0.02 or 0.05 mM GSSG or GSH.

Experiment 4: the effect of glutathione on the development of ICSI embryo

The oocytes, 10 h after the injection of hCG were collected from dissected fallopian tubes and washed with mR1ECM medium. And the cumulus-oocyte complexes were released from the oviducts into 80 IU/ml bovine testicular hyaluronidase (300 units/mg) in 22 mM Hepes-mR1ECM medium to disperse cumulus cells. The cumulus-free oocytes were washed with Hepes-mR1ECM medium and used immediately for ICSI. Epididymal spermatozoa were obtained from males 12–16 week of age. The cauda epididymis were removed from each animal and placed in a 1 ml drop of mR1ECM medium (capacitation drop) under oil. The epididymal contents were expressed from the cauda epididymis with needles, and the tissue was discarded. Spermatozoa were allowed to disperse for 2–3 min at room temperature. A sample of sperm suspension for ICSI was taken immediately after sperm dispersion. The ICSI was carried out as described by Kimura and Yanagimachi [3]. Briefly, a small drop of sperm suspension was mixed thoroughly with an equal volume of Hepes-mR1ECM containing 7% (w/v) polyvinyl pyrolidone (Nakalai Tesque, Kyoto, Japan) immediately before ICSI, which was performed using OLYMPUS Micromanipulators (OLYMPUS-ON3, OLYMPUS, Tokyo, Japan) with a piezo-electric actuator (PMM Controller, model PMAS-CT150; Prima Tech, Tsukuba, Japan). A single motile spermatozoon was drawn tail-first into the injection pipette and moved back and forth until the head-midpiece junction (the neck) was at the opening of the injection pipette. The head was separated from the midpiece by applying one or more piezo pulses. After discarding the midpiece and tail, the head was redrawn into the pipette and injected immediately into an oocyte. The ICSI was done in Hepes-mR1ECM within 1–2 h after oocyte collection. Sperm-injected oocytes were transferred into mR1ECM medium with 0.01 mM GSSS or GSH and cultured. The oocytes were examined approximately 6 h after ICSI for survival and activation. The oocytes were treated with 10 mM $SrCL_2$ in Ca^{2+}-free CZB medium [11] for 30 min.

Embryo culture

Embryos were cultured with each medium at 37°C in humidified air with 5% CO_2 in drops under oil. Embryos were observed daily under an inverted microscope, and the number of embryos reaching to the 4-cell, 8-cell, morula, blastocyst stage was recorded until 120 h.

Blastocyst attaining

For blastocyst attaining, the embryos at expanded and partially hatched stages were chosen and incubated in a 300 µl solution of PBS with 1% Triton X and 100 µg/ml propidium iodide up to 30 s. Blastocysts were immediately transferred into 300 µl of fixative containing 100% ethanol with 25 µg/ml Hoechst 33342 and were stored at 4°C overnight. The stained embryos were put into glycerol and mounted on the glass microscope slides. Then embryos were observed under fluorescence microscopy and the number of ICM (blue) and trophoblast (red) nuclei were counted and photographed.

Statistical analysis

The data are expressed as means ± S.E.M. of values. Statistical analyses were conducted using one-way ANOVA for repeated measurements followed by Fishers protected least significant difference test as post hoc tests (Statcel2 software; OMS publishing Inc, Japan). Differences were considered to be statistically significant at $P < 0.05$.

Results

The effect of glutathione on the intact embryo development is shown in Table 1. It appears that both of the GSSG and GSH adversely affected embryo development up to the morula stage. With the addition of 0.01 mM GSH, blastocyst development increased to 61.9%, where in control the development rate was 52.0%. The addition of 0.05 mM GSH showed significantly reduced rate of blastocyst development ($P < 0.05$). There was no remarkable difference between GSSG and GSH for the embryo development. In both of GSSG and GSH treatments, 0.01 mM concentration exhibited better blastocyst development than the others.

Effect of glutathione microinjection on the embryo development is shown in Table 2. Microinjection of glutathione (GSSG, GSH) increased embryo development at each stage of development than control. In the microinjected embryos, GSSG and GSH significantly increased embryo development from 4-cell to morula stage ($P < 0.05$) than that of control. The blastocyst development in 0.2 nM GSSG (47.8%) and GSH (58.1%) was significantly higher ($P < 0.05$) than that of the control (23.8%). There was a decrease in blastocyst development with the increase of GSSG or GSH concentration. The overall rate of development with GSH was slightly higher than the GSSG treatment for almost all of the developmental stages.

Effect of glutathione sham-injection on the embryo development is shown in Table 3. Compared to control (20.0%), all of the GSSG and GSH concentration improved blastocyst development, where 0.01 mM GSH (46.7%) improved significantly ($P < 0.05$). The rate of development from 4-cell to morula stage was significantly higher with GSSG and GSH treatment ($P < 0.05$), and there was a decrease in relation to the increasing concentration. Though there was no significant difference between GSSG and GSH treatment on embryo development, the addition of GSH in the culture media showed a higher rate of development.

Table 1 Effect of glutathione addition to culture medium on the rate of embryo development

Concentration (mM)	No. of embryos (%)					
	n	2-cell	4-cell	8-cell	Morula	Blastocyst
Control	25	25 (100.0)	20 (80.0)^{a,b}	15 (60.0)^{a,b}	14 (56.0)^{a,b}	13 (52.0)^{a}
GSSG						
0.01	20	20 (100.0)	20 (100.0)^{a}	18 (90.0)^{a}	15 (75.0)^{a}	10 (50.0)^{a,b}
0.02	19	18 (94.7)	17 (89.5)^{a,b}	12 (63.2)^{a,b}	12 (63.2)^{a}	8 (42.1)^{a,b}
0.05	19	19 (100.0)	16 (84.2)^{a,b}	8 (42.1)^{b}	6 (31.6)^{b}	6 (31.6)^{a,b}
GSH						
0.01	21	21 (100.0)	20 (95.2)^{a,b}	15 (71.4)^{a,b}	13 (61.9)^{a,b}	13 (61.9)^{a}
0.02	14	14 (100.0)	13 (92.9)^{a,b}	11 (78.6)^{a,b}	8 (57.1)^{a,b}	4 (28.6)^{a,b}
0.05	23	21 (91.3)	16 (70.0)^{b}	10 (43.5)^{b}	7 (30.4)^{b}	5 (21.7)^{b}

Values in *parentheses* indicate percentage. Data are expressed as of four replicates. Each embryo contains 0.35, 0.7 and 1.75 *pg* glutathione for 0.01, 0.02 and 0.05 mM treatment, respectively

[a, b] Values with different superscripts within the same column are significantly different ($P < 0.05$)

Table 2 Effect of glutathione microinjection on the rate of embryos at various stage of development

Concentration (mM)	No. of embryos (%)					
	n	2-cell	4-cell	8-cell	Morula	Blastocyst
Control	63	63 (100.0)[a]	28 (44.4)[d]	19 (30.2)[c]	17 (27.0)[c]	15 (23.8)[c]
GSSG						
0.2	69	69 (100.0)[a]	57 (82.6)[a,b]	40 (58.0)[a,b]	35 (50.7)[a b]	33 (47.8)[a,b]
0.5	69	67 (97.1)[b]	52 (75.4)[b]	38 (55.1)[b]	29 (42.0)[b]	26 (37.7)[b]
1.0	65	65 (100.0)[a]	32 (49.2)[c]	17 (26.2)[c]	9 (13.8)[d]	9 (13.8)[d]
GSH						
0.2	31	31 (100.0)[a]	29 (93.5)[a]	24 (77.4)[a]	21 (67.7)[a]	18 (58.1)[a]
0.5	29	29 (100.0)[a]	25 (86.2)[a,b]	17 (58.6)[a,b]	12 (41.3)[b]	8 (27.6)[b,c]
1.0	28	28 (100.0)[a]	21 (75.0)[b,c]	18 (64.3)[a]	12 (42.9)[b]	9 (32.1)[b,c]

Values in *parentheses* indicate percentage. Data are expressed as of four replicates. Each embryo contains 7, 17.5, and 35 *pg* glutathione for 0.2, 0.5 and 1.0 mM treatment, respectively

[a, b, c, d] Values with different superscripts within the same column are significantly different ($P < 0.05$)

Table 3 Effect of glutathione sham-injection on the rate of embryos on various stage of development

Concentration (mM)	No. of embryos (%)					
	n	2-cell	4-cell	8-cell	Morula	Blastocyst
Control	25	25 (100.0)	15 (60.0)[b]	9 (36.0)[b]	5 (20.0)[b]	5 (20.0)[b]
GSSG						
0.01	30	30 (100.0)	28 (93.3)[a]	21 (70.0)[a]	19 (63.3)[a]	11 (36.7)[a,b]
0.02	25	25 (100.0)	19 (76.0)[a,b]	15 (60.0)[a,b]	11 (44.0)[a,b]	9 (36.0)[a,b]
0.05	30	30 (100.0)	25 (83.3)[a]	15 (50.0)[a,b]	12 (33.3)[a,b]	8 (26.7)[a,b]
GSH						
0.01	30	30 (100.0)	28 (93.3)[a]	20 (66.7)[a]	17 (56.7)[a]	14 (46.7)[a]
0.02	20	20 (100.0)	16 (80.0)[a,b]	14 (70.0)[a]	10 (50.0)[a]	8 (40.0)[a,b]
0.05	28	28 (100.0)	23 (82.1)[a]	19 (67.9)[a]	14 (50.0)[a]	10 (35.7)[a,b]

Values in *parentheses* indicate percentage. Data are expressed as of four replicates. Each embryo contains 0.35, 0.7 and 1.75 *pg* glutathione for 0.01, 0.02 and 0.05 mM treatment, respectively

[a, b] Values with different superscripts within the same column are significantly different ($P < 0.05$)

The effect of glutathione after ICSI embryo development is shown in Table 4. Though, there was no significant difference between control and the treatments, the rate of blastocyst development with 0.01 mM GSH was higher than that of the others.

The effect of glutathione on the inner cell mass (ICM) and trophectoderm (TE) cell number is shown in Table 5. In the first experiment, a larger number of ICM, TE and total cells were observed with both of the 0.01 mM GSSG and GSH treatment. In the second experiment, treatment of 0.2 mM GSSG or GSH showed a significantly larger number of TE and total cells ($P < 0.05$). Though in the third experiment, compared to control there was a decrease in the number of cells with 0.01 mM GSSG treatment, a larger number of TE and total cells were observed with GSH of same concentration.

Discussion

Mammalian embryos undergo species-specific in vitro "blocks" to development; in the rat this block occurs at the 2-cell and 4-cell stages [5]. Two common components in culture media, phosphate and glucose, appear to be responsible for the developmental block of rat embryos [13, 14]. Rat R1ECM [12] was reduced NaCl concentration, adding 7.5 mM glucose, and omitting the amino acid in HECM-1. With further modification of R1ECM was observed by adding 20 amino acids (mR1ECM) [12]. We previously observed that development of blastocyst from rat 8-cell was observed in the concentration from 0.1 M to 0.01 mM of GSH and GSSG [15]. As that result, development to blastocysts was improved with 0.01 mM GSH and 0.05 mM GSSG. The present study shows that the

Table 4 Effect of glutathione on embryo development after ICSI

Concentration (mM)		No. of embryos (%)					
		n	2-cell	4-cell	8-cell	Morula	Blastocyst
Control		10	8 (80.0)	6 (60.0)	3 (30.0)	2 (20.0)	1 (10.0)
ICSI + GSSG	0.01	8	6 (75.0)	4 (50.0)	4 (50.0)	2 (25.0)	1 (12.5)
ICSI + GSH	0.01	10	8 (80.0)	5 (50.0)	4 (40.0)	3 (30.0)	2 (20.0)

Values in *parentheses* indicate percentage. Data are expressed as of four replicates. Each embryo contains 0.35 *pg* glutathione

Table 5 Effect of glutathione on cell number of blastocyst

Concentration (mM)	*n*	Cell number		
		ICM	TE	Total
Glutathione in medium				
Control	4	13.0 ± 1.0	26.3 ± 1.7	39.3 ± 0.9
GSSG 0.01	3	15.0 ± 1.0	28.0 ± 2.1	43.0 ± 2.1
GSH 0.01	3	14.0 ± 1.5	31.7 ± 2.2	45.7 ± 3.7
Glutathione injection				
Control	4	10.0 ± 0.4	19.5 ± 1.0[b]	29.5 ± 1.3[b]
GSSG 0.2	3	11.3 ± 0.9	24.0 ± 1.0[a]	35.3 ± 1.5[a]
GSH 0.2	5	11.2 ± 0.7	24.4 ± 0.9[a]	35.6 ± 1.4[a]
Glutathione sham-injection				
Control	4	8.8 ± 0.6	18.5 ± 2.5	27.3 ± 2.2
GSSG 0.01	4	8.3 ± 0.9	16.7 ± 2.0	25.0 ± 2.5
GSH 0.01	5	8.8 ± 0.6	21.8 ± 2.4	30.5 ± 2.9

Values in *parentheses* indicate percentage. Each value represents the mean ± SEM. Each embryo contains 0.35 and 0.7 *pg* glutathione for 0.01 and 0.02 mM treatment, respectively

[a, b] Values with different superscripts within the same column are significantly different ($P < 0.05$)

development rate of blastocyst was promoted by the addition of 0.01 mM GSH. An addition of glutathione to serum-free culture medium improves the development of mouse zygotes through the 2-cell block to the morula or blastocyst stage [16].

Gardiner and Reed [17] reported that embryos do not possess the capacity to synthesize glutathione de novo until the blastocyst stage of development. During normal embryo development, GSH concentration decreases 90% during the period from the unfertilized oocyte to the blastocyst stage [18]. Sawai et al. [19] indicated that GSH is critical to the protection of oocytes from oxidative damage and apoptosis, microtubule polymerization during oocyte meiosis, sperm pronucleus formation after fertilization, and early embryo development. In this experiment, microinjection of glutathione (GSSG, GSH) increased embryo development at each stage of development. We suggest that the development of the embryo was increased with the addition of glutathione into the culture medium and embryo.

The optimum injection concentration was 0.2 mM for both of the GSSG and GSH treatments to rat embryo. Furnus et al. [20] evaluated the intracellular GSH/GSSG content in cattle oocytes for 3.1 pmol/oocyte. GSH synthesis involves the constitutive amino acids, glycine (Gly), glutamine (Glu) and cysteine (Cys). Cys is a rate-limiting step in GSH synthesis by the γ-glutamyl cycle [21–24] and is transported into cells via transport system alanine-serine-cysteine (ASC). The ASC neutral amino acid transporters [25] exhibit the properties of the classical Na^{+}-dependent amino acid transport system [26–29]. ASC transporters have a high-affinity for alanine (Ala), serine (Ser), threonine (Thr) and Cys [26–29]. In addition, GSH/GSSG concentrations were significantly decreased when Cys was omitted of the incubation medium. But addition of Cys to the maturation medium, either with or without Gly and Glu supplementation resulted in an increase of GSH/GSSG content oocytes and cumulus cells [20]. Differences in culture conditions are known to contribute to differences in GSH status of cells and may explain the varying results.

The difference wasn't seen in our experiment by the concentration of GSSG and GSH. Glutathione is an intracellular thiol-containing compound that participates in defending the cell against oxidative stress by acting both as a non-enzymatic free radical scavenger and as a substrate for various enzymatic reactions such as glutathione peroxidase [30], which lowers intracellular concentrations of H_2O_2 and other hydroperoxides. Another enzyme important in the glutathione system is glutathione reductase, which regenerates GSH from GSSG at the expense of NADPH [31].

The development rate of the rat embryo decreased from 4-cell onwards. Griffiths et al. [32] reported that the proportion of blastocyst was lower after ICSI than after conventional IVF. We speculate that the damage of embryo cause delay or totally arrest development. Miller and Smith [33] observed no significant difference between ICSI and IVF for early embryo development. Motoishi et al. [34] indicated that the ICSI procedure is not detrimental to embryo development.

The number of ICM, TE and total cells were increased to the intact and punctured embryo by the addition of GSSG or GSH. Thus, GSSG and GSH addition is

effective for the culture of the damaged embryo. In this experiment, GSH concentration in the embryo rose by microinjected GSH, and development rate of embryo improved the possibility that high quality blastocyst could be obtained by this method. Dumoulin et al. [35] reported that for the number of cells there was no significant difference between ISCI and IVF embryos. Cell number alone does not reflect blastocyst quality since the embryonic cells must be appropriately allocated between the ICM and TE. ICM is probably the most predictive of continued developmental potential [36]. Miller and Smith [33] found a significant decrease in the number and percentage of ICSI-derived blastocyst stage embryos with defined ICM. Our experiment also demonstrates almost the same trend of embryo development.

In conclusion, the addition of glutathione to the culture media can improve early embryo development and is capable of repairing the damage of ICSI rat embryos.

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