Release of two-cell block by reduction of protein disulfide with thioredoxin from *Escherichia coli* in mice

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Summary. The development of mouse pronuclear-stage embryos in media containing various concentrations of thioredoxin was monitored and the influence of anti-thioredoxin immunoglobulin G (IgG) and heat-treated thioredoxin on the thioredoxin-induced effects was evaluated. A significant increase in the number of four-cell embryos (76.3%) and blastocysts (37.3%) was observed when embryos were cultured in the medium containing 50 µg thioredoxin ml⁻¹ compared with the rates (55.8 and 3.8%, respectively) in the basic medium. The number of blastocysts increased significantly to a maximum of 70.2% at 500 µg ml⁻¹. The biological activity of thioredoxin was evident after dialysis, but was markedly impaired by the addition of anti-thioredoxin IgG to the culture medium. Treatment at 60°C for 5 min did not affect the enzymatic and biological activity of thioredoxin. More severe heat treatment (121°C for 30 min) attenuated the enzymatic activity to 40% of its initial value and reduced the biological activity (number of blastocysts, from 77.8 to 51.6%).

These results indicate that the effect of thioredoxin on the two-cell block is due to the thioredoxin molecule itself, and suggest that disulfide formation within or between proteins resulting from oxidative stress is one of the major causes of the two-cell block.

Keywords: thioredoxin; embryo; mouse

Introduction

Mammalian preimplantation embryos exhibit retarded growth and developmental blockage *in vitro* (Wright & Bondioli, 1981; Fisher, 1987). For example, in mice, embryos of outbred strains exhibit the developmental block at the two-cell stage *in vitro* (Whitten, 1957; Yanagimachi & Chang, 1964; Whittingham, 1975). In humans, eggs fertilized *in vitro* show retarded growth at the four-cell and more advanced stages and few embryos grow to the blastocyst stage (Fishel, 1986). These phenomena are thought to be caused by deficiencies of the conventional culture system compared with the *in vivo* environment; neither growth retardation nor developmental blockage are observed *in vivo*.

Oxygen concentration *in vitro* (i.e. the atmosphere) is about 3–10 times higher than that *in vivo* (i.e. oviduct or uterus) (Bishop, 1956; Mastroianni & Jones, 1965; Maas *et al.*, 1976). Low oxygen tension of culture media has been reported to promote embryo development in many species (Whitten, 1970). We have shown that the two-cell block in mice is released by adding superoxide dismutase (SOD), a scavenger of superoxide anion radicals, to the culture medium (Noda *et al.*, 1991). An additive effect of low oxygen and SOD on mouse embryo development has been demonstrated (Umaoka *et al.*, 1992). Generation of reactive oxygen species in blocked embryos (Nasr-Esfahani *et al.*, 1990a) and overcoming the two-cell block by apotransferrin and iron chelators, which suppress the Haber-Weiss reaction (Nasr-Esfahani *et al.*, 1990b), have also been reported. Considering these results together, it is possible that the two-cell block in mice is due to damage to the embryos by oxygen radicals.

It is known that oxygen radicals inactivate various enzymes and damage membranes and DNA (Halliwell & Gutteridge, 1989). The sulfhydryl group of proteins, readily oxidized under oxidative stress (Brigelius, 1985), may be damaged by oxygen radicals resulting in blocked embryo development *in vitro*. Recently, thioredoxin (Laurent *et al.*, 1964), a small heat-resistant enzyme (molecular mass of about 12 kDa), which promotes the redox reaction of sulfhydryl groups of proteins, was demonstrated to have a defensive role against oxidative stress (Holmgren, 1985). In this study the effects of thioredoxin on mouse embryo development were evaluated and the specificity of the enzyme was established by maintaining mouse embryo cultures in media containing thioredoxin, anti-thioredoxin IgG or heat-treated thioredoxin.

Materials and Methods

Chemicals and enzymes

Thioredoxin (from *Escherichia coli*) was purchased from Promega Co. (Madison, WI, USA). Before use, the thioredoxin preparation was dialysed extensively against distilled water followed by lyophilization. Pregnant mares serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) were purchased from Teikoku Zoki Co. (Tokyo, Japan). Bovine serum albumin (BSA), hyaluronidase (bovine) and insulin (bovine) were purchased from Sigma Chemical Co. (MO, USA). The other chemicals were of reagent grade and purchased from Nacalai Tesque Co. (Kyoto, Japan).

Preparation of anti-thioredoxin antiserum and extraction of the IgG fraction

The anti-thioredoxin antiserum was prepared by injecting the immunogen (0.5 mg ml^{-1}), with Freund's complete adjuvant, into the dorsal skin of female rabbits six times at intervals of 2 weeks. The rabbits were bled 7 days after the last injection. The IgG fractions of anti-thioredoxin antiserum (α -Trx-IgG) and normal rabbit serum (NRS-IgG) were recovered from a protein-A Sepharose 4B column (Duhamel *et al.*, 1979). The IgG preparations were dialysed against distilled water and lyophilized.

Reactivity of α -Trx-IgG was examined by the method of Ouchterlony & Nilson (1978). Thioredoxin, BSA, human albumin, mouse liver homogenate and α -Trx-IgG were added to the diffusion well and incubated at 4°C overnight. The formation of precipitation bands was examined.

Heat treatment of thioredoxin

Thioredoxin was dissolved in deionized water at 1 mg ml^{-1} and the solution was divided into three parts, which were then heat-treated (i) in a hot waterbath at 60°C for 5 min, (ii) at 100°C for 30 min, or (iii) in an autoclave at 121°C for 30 min. The heat-treated thioredoxin specimens were freeze-dried and stored.

Enzyme activity of the heat-treated thioredoxin was assayed by a turbidimetric assay using dithiothreitol (DTT) and insulin (Holmgren, 1979). The heat-treated thioredoxin samples were dissolved in phosphate buffer (0.1 mol l^{-1}) and then added to the reaction cuvette (volume 1.2 ml) at a final concentration of $60 \mu\text{g ml}^{-1}$ ($5 \mu\text{mol l}^{-1}$). Reaction media contained 0.13 mmol l^{-1} insulin, 0.33 mmol l^{-1} DTT, 2 mmol l^{-1} EDTA and 0.1 mol l^{-1} phosphate buffer. Increase in absorbance at 650 nm (A_{650}) caused by splitting of insulin molecules was recorded using a Shimadzu UV-260 spectrophotometer. Thioredoxin activity was evaluated using the maximum increase in A_{650} in one minute. Standard curves were made from original samples of thioredoxin at 0, 2.5, 5, 7.5 and $10 \mu\text{mol l}^{-1}$.

Embryo collection and culture conditions

TUCK (outbred) mice were purchased from A. Tuck and Son Ltd (Battlesbridge, UK). Female mice, aged 4–5 weeks, were injected i.p. with PMSG (5 iu) and hCG (5 iu) at an interval of 48 h to induce superovulation. They were then mated with 12-week-old male mice of the same strain. Vaginal plug formation was confirmed on the next morning (day 1 of pregnancy). Female mice were killed by cervical dislocation, and pronuclear-stage embryos were collected from the ampulla of oviducts by the scratching method 17 h after hCG injection. After the removal of cumulus cells with 0.1% hyaluronidase, all the embryos were pooled in an $80 \mu\text{l}$ spot of Dulbecco's phosphate saline, and 10–18 embryos with normal morphology were placed into each experimental spot of medium at random and then cultured at 37°C under $5\% \text{ CO}_2$ in air. Degenerated embryos were excluded.

The basic medium for embryo culture was Biggers–Whitten–Whittingham (BWW) solution (Biggers *et al.*, 1971) supplemented with 0.3% BSA. An $80 \mu\text{l}$ spot of medium was placed in each well of the four-well multidish (Nunc Co., Denmark) and covered with mineral oil. Embryos were observed every 24 h under an Olympus IMT-2 microscope with a Nomarski differential interferometer. The culture efficacy in the following experiments was evaluated by determining the proportion of embryos reaching two-cell (day 2), four-cell (day 3) and blastocyst (day 5) stages.

Experiment 1: embryo culture in the thioredoxin-supplemented medium

Pronuclear-stage embryos were cultured after adding various concentrations (10, 50, 100, 500 and 1000 $\mu\text{g ml}^{-1}$) of thioredoxin to the basic medium. As a control, pronuclear embryos were cultured in the basic medium. Embryo cultures in each thioredoxin-supplemented medium were started at the same time.

Experiment 2: inhibition of thioredoxin-induced effects by α -Trx-IgG

Pronuclear-stage embryos were cultured in the following media: group 1, basic medium containing thioredoxin ($30 \mu\text{g ml}^{-1}$, $2.5 \mu\text{mol l}^{-1}$); group 2, basic medium containing thioredoxin ($30 \mu\text{g ml}^{-1}$) and NRS-IgG ($500 \mu\text{g ml}^{-1}$); group 3, basic medium containing thioredoxin ($30 \mu\text{g ml}^{-1}$) and α -Trx-IgG ($500 \mu\text{g ml}^{-1}$); group 4, basic medium containing NRS-IgG ($500 \mu\text{g ml}^{-1}$); group 5, basic medium containing α -Trx-IgG ($500 \mu\text{g ml}^{-1}$); and group 6, basic medium. Embryo cultures in the six groups were started at the same time.

Experiment 3: influence of heat treatment on thioredoxin-induced effects

Pronuclear-stage embryos were cultured after adding heat-treated thioredoxin at $500 \mu\text{g ml}^{-1}$ ($41.7 \mu\text{mol l}^{-1}$) to the basic medium. As a control, pronuclear-stage embryos were cultured in the basic medium containing untreated thioredoxin ($500 \mu\text{g ml}^{-1}$). Embryo cultures in each medium were started at the same time.

Statistical analysis

The results of each experiment were analysed by the χ^2 test.

Results

Reactivity of α -Trx-IgG was examined by Ouchterlony's method. A single precipitation band was observed between α -Trx-IgG ($80 \mu\text{g}$) and thioredoxin ($1 \mu\text{g}$) (Fig. 1). No precipitation band was observed against BSA, human albumin and mouse liver homogenate at various amounts (0.1, 1, 10 and 100 μg).

Fig. 1. Precipitation pattern between the immunoglobulin fraction of anti-thioredoxin rabbit antiserum (α -Trx-IgG) and thioredoxin. A single precipitation band is observed between α -Trx-IgG (A: $80 \mu\text{g}$) and thioredoxin (C: $1 \mu\text{g}$). No precipitation bands are found between α -Trx-IgG ($80 \mu\text{g}$) and BSA (B: $1 \mu\text{g}$), human albumin (D: $1 \mu\text{g}$), or mouse liver homogenate (E: $1 \mu\text{g}$).

The enzyme activity of heat-treated thioredoxin solution remained high after 5 min at 60°C (Table 1). Heat treatment at 100°C for 30 min and at 121°C for 30 min decreased enzymatic activity to 80% and 40% of that of untreated thioredoxin, respectively.

Table 1. Turbidimetric assay of thioredoxin

Specimens	Enzymatic activity ($\Delta A_{650} \text{ nm min}^{-1}$)
Thioredoxin ($\mu\text{mol ml}^{-1}$)	
0	0.000
2.5	0.067
5.0	0.093
7.5	0.149
10.0	0.178
Heat-treated thioredoxin ($5.0 \mu\text{mol ml}^{-1}$)	
60°C, 5 min	0.088
100°C, 30 min	0.075
121°C, 30 min	0.039

Effects of thioredoxin on embryo development

A significant increase in the numbers of embryos at the four-cell (day 3) (76.3%) and blastocyst (day 5) (37.3%) stages was observed when the culture media contained $50 \mu\text{g}$ thioredoxin ml^{-1} ($4.2 \mu\text{mol l}^{-1}$), compared with culture in the basic medium (55.8%, 3.8%). When the concentration was increased, the frequency of blastocysts also increased and then decreased gradually at higher concentrations (Table 2).

Table 2. Effects of thioredoxin on mouse embryo development

Conditions	Number of trials	Number of embryos examined ^a	Number (%) ^b of embryos developed to		
			two-cell stage	four-cell stage	blastocyst stage
Basic medium + thioredoxin ($\mu\text{g ml}^{-1}$)					
10	4	60	50 (83.3)	31 (51.7)	9 (15.0)
50	4	59	52 (88.1)	45 (76.3)*	22 (37.3)**
100	4	59	54 (91.5)	44 (74.6)*	35 (59.3)**
500	4	57	49 (86.0)	48 (84.2)**	40 (70.2)**
1000	4	55	46 (83.6)	43 (78.2)*	34 (61.8)**
5000	4	57	52 (91.2)	46 (80.7)**	31 (54.4)**
Basic medium (control)	4	52	46 (88.5)	29 (55.8)	2 (3.8)

^aPronuclear-stage embryos collected 17 h after administration of hCG. 12–16 embryos were used per experiment.

^bPercentages of embryos at each stage represent cumulative totals.

* $P < 0.05$; ** $P < 0.01$, compared with the control value.

Inhibition of thioredoxin-induced effects by α -Trx-IgG

Flocculate precipitates were observed 12 h after the addition of α -Trx-IgG to the medium containing thioredoxin, but not otherwise.

A significantly higher incidence of blastocysts was obtained by culture with thioredoxin alone (28.1%, group 1) or with thioredoxin and 500 μg NRS-IgG ml^{-1} (28.8%, group 2), compared with that (4.1%) noted in the basic medium (group 6). However, the incidence of blastocysts in the medium containing thioredoxin and α -Trx-IgG in group 3 was low (10.7%) and comparable with that found in the basic medium. The addition of NRS-IgG or α -Trx-IgG alone to the basic medium did not significantly affect the incidence of blastocysts (groups 4 or 5) (Table 3).

Table 3. Inhibition of thioredoxin-induced effects by α -thioredoxin-IgG in mice

Conditions	Number of trials	Number of embryos examined ^a	Number (%) ^b of embryos developed to		
			two-cell stage	four-cell stage	blastocyst stage
BWW + Trx	4	57	51 (89.5)	40 (70.2)*	16 (28.1)**
BWW + Trx + NRS-IgG	4	56	51 (98.1)	27 (51.9)	15 (28.8)**
BWW + Trx + α -Trx-IgG	4	52	53 (94.6)	15 (26.8)	6 (10.7)
BWW + NRS-IgG	4	60	59 (98.3)	22 (36.6)	1 (1.7)
BWW + α -Trx-IgG	4	63	62 (98.4)	23 (36.5)	4 (6.3)
BWW (control)	4	49	45 (91.8)	23 (46.9)	2 (4.1)

^aPronuclear-stage embryos collected 17 h after administration of hCG. 10–17 embryos were used per experiment.

^bPercentages of embryos at each stage represent cumulative totals.

BWW: Biggers–Whitten–Whittingham solution.

Trx: Thioredoxin from *Escherichia coli* at 30 μg ml^{-1} .

NRS-IgG: IgG fraction of normal rabbit serum at 500 μg ml^{-1} .

α -Trx-IgG: IgG fraction of anti-thioredoxin antiserum at 500 μg ml^{-1} .

* $P < 0.05$; ** $P < 0.01$, compared with the control values.

Influence of heat treatment on thioredoxin-induced effects

A significant increase in the incidence of both the four-cell and blastocyst stages was observed in the culture media containing each heat-treated thioredoxin or untreated thioredoxin, compared with the incidence in the basic medium. The embryo promoting effect of thioredoxin was not influenced significantly by heat treatment at 60°C for 5 min or at 100°C for 30 min, but was impaired by heat treatment at 121°C for 30 min (Table 4).

Discussion

In this study, we demonstrated that thioredoxin overcomes the two-cell block in mice and promotes the development of mouse embryos *in vitro*. The biological activity of thioredoxin was not lost after dialysis, but was markedly impaired after the addition of α -Trx-IgG to the culture medium, indicating that the effect of the thioredoxin preparation on the two-cell block phenomenon is due to the thioredoxin molecule itself. Heat treatment at 60°C for 5 min did not diminish the enzymatic and biological activity of thioredoxin, showing the extraordinary heat stability of this enzyme (Laurent *et al.*, 1964; Holmgren, 1985). Heat treatment at 121°C for 30 min reduced the enzymatic activity to 40% of initial values and decreased the biological activity even more, suggesting that the promotion of embryo development by thioredoxin is a phenomenon dependent on the enzyme activity of thioredoxin.

Table 4. Influence of the heat treatment on thioredoxin-induced effects in mice

Conditions	Number of trials	Number of embryos examined ^a	Number (%) ^b of embryos developed to		
			two-cell stage	four-cell stage	blastocyst stage
BWW + Trx (60°C, 5 min) ^c	4	61	58 (95.1)	53 (86.9)	45 (73.8)
BWW + Trx (100°C, 30 min)	4	64	60 (93.8)	51 (79.7)	46 (71.9)
BWW + Trx (121°C, 30 min)	4	64	61 (95.3)	53 (82.8)	33 (51.6)*
BWW + Trx ^d	4	63	59 (93.7)	55 (87.3)	49 (77.8)
BWW	4	65	62 (95.4)	35 (53.8)	3 (4.6)

^aPronuclear-stage embryos collected 17 h after administration of hCG; 13–18 embryos were used per experiment.

^bPercentages of embryos at each stage represent cumulative totals.

^cHeat-treated thioredoxin at 500 µg ml⁻¹.

^dUntreated thioredoxin at 500 µg ml⁻¹.

**P* < 0.05, compared with the control (BWW + Trx).

BWW: Biggers–Whitten–Whittingham solution; Trx: thioredoxin.

Many investigators have studied the *in vitro* two-cell block in mice since it was first reported by Whitten (1957). In an early study, a low oxygen concentration (5% O₂) was found to enhance the development of mouse embryos (Whitten, 1970). Recently, SOD, a scavenger of superoxide anion radicals, was shown to further enhance the effect of low oxygen concentrations on mouse embryo development (Noda *et al.*, 1991) and an additive effect of low oxygen concentration and SOD on mouse embryo development was also described by Umaoka *et al.* (1992). It is therefore possible that the mouse two-cell block is primarily caused by damage to the embryo by reactive oxygen species generated in high oxygen tension media.

In general, with oxidative stress, the rates of disulfide bond formation and mixed disulfide formation increase within the cell (Brigelius, 1985; Park & Thomas, 1989), resulting in the inactivation of enzymes, and reactive oxygen species are involved in protein disulfide formation (Halliwell & Gutteridge, 1989). Thioredoxin is known to play a defensive role against oxidative stress by reducing protein disulfide bonds. For example, in higher plants, thioredoxin reactivates the oxidized enzymes involved in photosynthesis (Schurmann *et al.*, 1981; Crawford *et al.*, 1986; Jacquot & Deottingnies 1986; Clancey & Gilbert, 1987), and, in animals, ornithine decarboxylase is known to be repaired by thioredoxin (Flamigni *et al.*, 1989). In mouse embryos, thioredoxin is also thought to promote development *in vitro* by facilitating the repair of oxidative changes of sulfhydryl groups.

Thioredoxin is a heat-stable small protein that is a hydrogen donor of *Escherichia coli* ribonucleotide diphosphate reductase and this protein is found in many prokaryotes and eukaryotes (Holmgren, 1985). The amino acid sequence of its active site is Cys-Gly-Pro-Cys and the two cysteine residues take part in a thiol–disulfide exchange reaction with a substrate (Holmgren, 1985). The thioredoxin that we added to the medium was an oxidized form, because only oxidized thioredoxin can be obtained from *E. coli* (Holmgren, 1985). As only the reduced form of thioredoxin is biochemically active and as culture media alone cannot reduce thioredoxin, the enzyme may be incorporated in the embryo where it is reduced by thioredoxin reductase using NADPH (Moore *et al.*, 1964; Thelander, 1967). The incorporation of very small amounts of thioredoxin into lens cells has also been demonstrated (Spector *et al.*, 1988).

The mechanism by which oxidative stress damages embryos remains to be established. The number of genomes of two-cell embryos blocked *in vitro* is 4n (Goddard & Pratt, 1983) and the embryos arrest after the S phase of the cell cycle (Luthardt & Donahue, 1975; Goddard & Pratt,

1983), suggesting that a system that regulates the cell cycle from the S to M phase or a factor involved in cell division is damaged in the two-cell embryos blocked *in vitro*. The release of mouse two-cell block by thioredoxin suggests that the sulfhydryl group of proteins involved in cell division or cell cycle regulation is one of the targets of oxidative stress. For example, tubulin may be a target, because the sulfhydryl group in the cystine residue of tubulin, which is a component of the microtubules in the mitotic apparatus, is involved in the polymerization of tubulin to form the microtubules (Kuriyama & Sakai, 1974).

In conclusion, we have demonstrated that thioredoxin releases mouse two-cell block and promotes the *in vitro* development of mouse embryos, and that the effects were induced by the thioredoxin molecule itself. Taking into consideration the fact that low oxygen concentration and SOD also promote development of embryos, we suggest that embryos must be carefully protected from oxidative stress *in vitro*. The precise mechanism of action of thioredoxin remains to be determined.

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