Alleviation of the two-cell block of ICR mouse embryos by polyaminocarboxylate metal chelators

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The present study was undertaken to examine the effects of various transition metal ion chelators, both polyaminocarboxylates (including nitrilotriacetate (NTA), ethylenediaminediacetate (EDDA), ethyleneglycolbistetraacetate (EGTA), ethylenediaminetetraacetate (EDTA) and diethylenetriaminepentaacetate (DTPA)) and non-polyaminocarboxylates (dipicolinic acid and deferoxamine), on the development *in vitro* of one-cell ICR strain mouse embryos to the four-cell and blastocyst stages. The order of stability constants of polyaminocarboxylates for transition metal ions such as zinc, copper and iron is as follows: $\text{NTA} \leq \text{EDDA} < \text{EGTA} < \text{EDTA} < \text{DTPA}$. Addition of 10 or 100 μmol polyaminocarboxylates l^{-1} to the medium significantly enhanced the development of most one-cell embryos (66–88%) beyond the two-cell stage compared

with that (< 25%) in medium without polyaminocarboxylates. Although EDDA, EDTA and DTPA at 10 μmol l^{-1} induced the development of most one-cell embryos to the four-cell stage and beyond, a higher concentration (100 μmol l^{-1}) of NTA and EGTA was required to obtain a similar result. Therefore, the ability of polyaminocarboxylates to overcome the two-cell block is not correlated with their potency to chelate transition metal ions. In contrast, the non-polyaminocarboxylates dipicolinic acid and deferoxamine, at 10 and 100 μmol l^{-1} , did not have the same effect. Taken together, the results indicate that the ability of polyaminocarboxylates to overcome the two-cell block in embryo development is due to some common feature or features other than the ability to chelate transition metal ions.

Introduction

In vitro culture of one-cell embryos from most strains of mouse is known to result in developmental arrest during the G2 phase of the second cell cycle, when the major zygotic gene activation occurs. This developmental arrest is referred to widely as the 'two-cell block'. In some strains, such as the ICR strain, the developmental block is alleviated by adding ethylenediaminetetraacetate (EDTA) to the culture medium, resulting in an increased proportion of one-cell embryos that develop to the blastocyst stage (Abramczuk *et al.*, 1977; Hoshi and Toyoda, 1985; Chatot *et al.*, 1989; Lawitts and Biggers, 1992; Gardner and Lane, 1996). As EDTA is a cell membrane-impermeable metal ion chelator, the effects of EDTA may be exerted by chelating extracellular transition metal ions such as zinc, copper and iron. For example, chelation of transition metal ions by EDTA generally prevents the ions from participating in chemical reactions that generate harmful oxygen radicals (Halliwell and Gutteridge, 1984). Inclusion of superoxide dismutase (SOD) in culture medium without EDTA partially alleviates the two-cell block, although the effects of SOD are disputed and variable (Legge and Sellens, 1991; Noda *et al.*, 1991; Payne *et al.*, 1992; Johnson and Nasr-Esfahani, 1994). Thus, one possible role of EDTA in the culture medium is to act as

a chelator of transition metal ions to prevent extracellular generation of oxygen radicals. However, although polyaminocarboxylate metal chelators, including EDTA, nitrilotriacetate (NTA), ethylenediaminediacetate (EDDA), ethyleneglycolbistetraacetate (EGTA), and diethylenetriaminepentaacetate (DTPA), are commonly used to prevent metal-catalysed oxidation because they sequester metals, a number of studies have indicated that iron- or copper-polyaminocarboxylate complexes may be catalytically active as a result of their specific co-ordination geometry (Yamada *et al.*, 1987; Stadtman, 1993; Asaumi *et al.*, 1996; Okada, 1996; Zhao *et al.*, 1996). Production of oxygen radicals, such as hydroxyl radicals, in the presence of polyaminocarboxylates tends to increase with decreased complex stability: $\text{NTA} \geq \text{EDDA} \geq \text{EDTA} > \text{DTPA}$ (Asaumi *et al.*, 1996; Okada, 1996; Zhao *et al.*, 1996). Furthermore, it has been reported that iron-chelated EDTA (Fe-EDTA) plays a catalytic role and promotes the oxidation of amino acids by the classical Fenton system (Asaumi *et al.*, 1996; Zhao *et al.*, 1996). Moreover, although the metal ion complexes formed with EDTA and EGTA have similar stability constants, Hoshi and Toyoda (1985) reported that EGTA did not alleviate the two-cell block in embryo development. Thus, it remains unclear whether the alleviation effect of EDTA is mediated by its ability to chelate transition metal ions, resulting in either sequestration or generation of reactive oxygen radicals.

The aim of the present study was to examine the effects

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Table 1. Dose effects of ethylenediaminetetraacetate (EDTA) on development of one-cell ICR mouse embryos *in vitro*

EDTA ($\mu\text{mol l}^{-1}$)	Number of embryos examined	Percentage of embryos developed to		
		Two-cell stage	Four-cell stage	Blastocyst
0.0	47	100 ^a	24 ^a	3 ^a
0.25	50	100 ^a	46 ^b	21 ^{ab}
0.5	55	100 ^a	75 ^c	37 ^b
1.0	55	100 ^a	84 ^c	59 ^b
10	56	100 ^a	92 ^c	82 ^c
100	38	100 ^a	95 ^c	85 ^c
1000	53	100 ^a	64 ^c	3 ^a

^{abc}Values in the same column with different superscripts are significantly different.

of polyaminocarboxylates and other chelators (non-polyaminocarboxylates) with different metal ion stability constants on the two-cell block in cultured ICR mouse embryos.

Materials and Methods

Chemicals

The chemicals used were obtained as follows: NTA, EDDA, EDTA, EGTA, DTPA, and Ca^{2+} - or Mg^{2+} -saturated EDTA (Ca-EDTA and Mg-EDTA, respectively) from Dojindo Laboratories (Kumamoto); dipicolinic acid from Molecular Probes (Eugene, OR); and deferoxamine from Sigma (St Louis, MO).

Embryo collection and culture

Female ICR mice, aged 3–6 weeks, were superovulated by injection of 5 iu equine chorionic gonadotrophin (eCG) followed 48 h later by 5 iu human chorionic gonadotropin (hCG). The females were subsequently mated with male mice of the same strain, and vaginal plug formation was confirmed on the next morning (day 1). The female mice were anaesthetized with ether and killed by cervical dislocation. All procedures involving animals were approved by the Kyoto University Animal Care and Use Committee. Fertilized one-cell embryos were collected 20 h after administration of hCG from the ampullae of oviducts of superovulated females by tearing the ampullae with a hypodermic needle. After removal of cumulus cells by digestion with 0.1% (w/v) hyaluronidase (Sigma) for approximately 5 min, the embryos were placed in 50 μl drops of culture medium, covered with mineral oil and cultured for 5 days at 37°C under 5% CO_2 in air. The embryos were observed every 24 h under a Nikon inverted microscope. The culture efficiency was evaluated by determining the proportion of embryos reaching the two-cell (day 2), four-cell (day 3) and blastocyst (day 5) stages.

The basal culture medium used was potassium simplex optimized medium (KSOM; Erbach *et al.*, 1994) without

EDTA, supplemented with 0.1% (w/v) polyvinylpyrrolidone instead of BSA and designated here as modified KSOM (mKSOM).

Statistical analysis

Each experiment was repeated three times. Subclass means were analysed by ANOVA and Scheffe's test. Percentage data were subjected to arcsine transformation before statistical analysis. A value of $P < 0.05$ was considered to be an indication of significance.

Results

The first experiment examined the effects of EDTA at various concentrations on development *in vitro* of one-cell embryos at the pronuclear stage to the four-cell and blastocyst stages. When concentrations of EDTA $> 0.25 \mu\text{mol l}^{-1}$ were added to mKSOM, the proportions of embryos reaching the four-cell and blastocyst stages increased significantly in a dose-dependent manner (Table 1). The prominent effects of EDTA on development to the four-cell and blastocyst stages were observed at concentrations of 0.5–100.0 $\mu\text{mol l}^{-1}$ and 10–100 $\mu\text{mol l}^{-1}$, respectively (the rates of development to the four-cell and blastocyst stages were 75–92% and 80–82%, respectively). However, addition of 1 mmol EDTA l^{-1} to the medium significantly reduced the proportion of embryos that developed to the four-cell stage, and the resulting four-cell embryos did not develop to the blastocyst stage. In the control group, in which the embryos were cultured in mKSOM without EDTA, the developmental rates to the four-cell and blastocyst stages were 24% and 3%, respectively.

Lane and Gardner (2001) suggested that the alleviation effect of EDTA is due to the chelation of divalent cations such as magnesium. Therefore, the next experiment examined the effects of Mg-EDTA and Ca-EDTA on development of one-cell embryos *in vitro*. Mg-EDTA and Ca-EDTA are non-magnesium and non-calcium chelators that are able to bind to metal ions with higher stability

Table 2. Effects of Ca- and Mg-ethylenediaminetetraacetate (EDTA) on development of one-cell ICR mouse embryos *in vitro*

Additive	Concentration ($\mu\text{mol l}^{-1}$)	Number of embryos examined	Percentage of embryos developed to		
			Two-cell stage	Four-cell stage	Blastocyst
–	–	65	100 ^a	14 ^a	0 ^a
Ca-EDTA	10	37	100 ^a	91 ^b	69 ^b
	100	40	100 ^a	89 ^b	73 ^b
Mg-EDTA	10	38	100 ^a	97 ^b	79 ^b
	100	38	100 ^a	98 ^b	74 ^b

^{abc}Values in the same column with different superscripts are significantly different.

Table 3. Stability constants of chelators for transition metal ions

Chelator	Log stability constant for				
	Zn ²⁺	Cu ²⁺	Fe ³⁺	Ca ²⁺	Mg ²⁺
NTA ^a	10.7	13.0	15.9	6.4	5.5
EDDA ^a	11.2	16.2	17.0	na	3.9
EGTA ^a	14.5	17.8	20.5	11.0	5.2
EDTA ^a	16.5	18.8	25.1	11.0	8.7
DTPA ^a	18.8	21.5	28.6	10.7	9.3
Dipicolinic acid ^b	6.4	9.1	na	4.4	2.3
Deferoxamine ^c	11.1	14.1	30.6	2.6	na

^aData from Dojindo Laboratories, Kumamoto, Japan.

^bData from Sakaguchi and Ueno (1967).

^cData from Imamura (1977).

DTPA: diethylenetriaminepentaacetate; EDDA: ethylenediaminediacetate; EDTA: ethylenediaminetetraacetate; EGTA: ethyleneglycolbistetraacetate; NTA: nitrilotriacetate; na: not available.

constants than magnesium and calcium ions, respectively. Mg-EDTA and Ca-EDTA at 10 and 100 $\mu\text{mol l}^{-1}$ had similar effects to 10 and 100 $\mu\text{mol l}^{-1}$ EDTA in alleviation of the two-cell block (Table 2). This result indicates that the effect of EDTA is not affected by the binding of magnesium or calcium ions to EDTA.

The ability of various polyaminocarboxylates (homologous ethyleneamine acetates) with different stability constants (Table 3) and non-polyaminocarboxylate chelators to alleviate the two-cell block was compared. All the polyaminocarboxylates examined had the ability to overcome the two-cell block (Table 4). Most embryos ($\geq 95\%$) treated with EDDA, EDTA or DTPA at 10 or 100 $\mu\text{mol l}^{-1}$ developed to the four-cell stage and subsequently to the blastocyst stage. However, in the embryos treated with NTA or EGTA at 10 $\mu\text{mol l}^{-1}$, no significant increase in the proportions of embryos at the four-cell and blastocyst stages was observed compared with embryos cultured in the basal medium. However, both NTA and EGTA at 100 $\mu\text{mol l}^{-1}$ alleviated the two-cell block. The proportion of embryos reaching the four-cell stage was significantly lower in the EGTA-treated embryos (66%), but not in the NTA-treated embryos (88%), than in embryos treated with other polyaminocarboxylates ($\geq 95\%$). The rates of development to

the blastocyst stage of embryos treated with NTA or EGTA at 100 $\mu\text{mol l}^{-1}$ (74% and 48%, respectively) were also lower compared with those ($\geq 89\%$) of embryos treated with other polyaminocarboxylates.

The effects of dipicolinic acid and deferoxamine, non-polyaminocarboxylates, on the development of one-cell embryos are summarized (Table 5). Dipicolinic acid has a lower stability constant for zinc, copper and iron than any of the polyaminocarboxylates (Table 3), but is generally used as a selective zinc chelator (Yamaguchi and Matsui, 1989; Larson and Kitto, 1999). Deferoxamine has a similar stability constant to EDDA for zinc and copper, but has the highest stability constant for iron among the chelators examined, and is generally used as a selective iron chelator (Nasr-Esfahani *et al.*, 1990). In embryos treated with 10 and 100 $\mu\text{mol l}^{-1}$ dipicolinic acid, the rates of development to the four-cell (6% and 27%, respectively) and blastocyst (0% and 3%, respectively) stages were not significantly different from those in the untreated control embryos (four-cell stage embryos, 6% and blastocysts, 0%). However, when one-cell embryos were treated with 1 or 10 $\mu\text{mol l}^{-1}$ deferoxamine, the cleavage rates (73% and 53%, respectively) to the two-cell stage were significantly lower than those (100%) of the control embryos; moreover, deferoxamine did not overcome

Table 4. Effects of polyaminocarboxylates on development of one-cell ICR mouse embryos *in vitro*

Additive	Concentration ($\mu\text{mol l}^{-1}$)	Number of embryos examined	Percentage of embryos developed to		
			Two-cell stage	Four-cell stage	Blastocyst
–	–	50	100 ^a	0 ^a	0 ^a
NTA	10	52	100 ^a	22 ^a	2 ^a
	100	53	100 ^a	88 ^b	74 ^b
EDDA	10	52	100 ^a	98 ^b	97 ^b
	100	51	100 ^a	98 ^b	96 ^b
EGTA	10	50	100 ^a	4 ^a	0 ^a
	100	52	100 ^a	66 ^c	48 ^c
EDTA	10	51	100 ^a	95 ^b	89 ^b
	100	52	100 ^a	98 ^b	98 ^b
DTPA	10	52	100 ^a	100 ^b	97 ^b
	100	51	100 ^a	98 ^b	96 ^b

DTPA: diethylenetriaminepentaacetate; EDDA: ethylenediaminediacetate; EDTA: ethylenediaminetetraacetate; EGTA: ethyleneglycolbistetraacetate; NTA: nitrilotriacetate.

^{abc}Values in the same column with different superscripts are significantly different.

Table 5. Effects of dipicolinic acid and deferoxamine on development of one-cell ICR mouse embryos *in vitro*

Additive	Concentration ($\mu\text{mol l}^{-1}$)	Number of embryos examined	Percentage of embryos developed to		
			Two-cell stage	Four-cell stage	Blastocyst
–	–	105	100 ^a	6 ^a	0 ^a
Dipicolinic acid	10	62	100 ^a	6 ^a	0 ^a
	100	64	100 ^a	27 ^a	3 ^a
Deferoxamine	1	48	73 ^b	7 ^a	0 ^a
	10	49	53 ^b	0 ^a	0 ^a

^{ab}Values in the same column with different superscripts are significantly different.

the two-cell block at either concentration. Thus, deferoxamine, at the concentrations examined, is deleterious to embryo development during the early cleavage stages.

When one-cell embryos were cultured in medium supplemented with both deferoxamine and EDTA, each at $10 \mu\text{mol l}^{-1}$ (Table 6), almost the same proportion of embryos developed to the four-cell stage as when the embryos were treated with $10 \mu\text{mol EDTA l}^{-1}$ alone. However, development to the blastocyst stage was completely inhibited in embryos treated with both chelators. These results indicate that the effect of EDTA on the two-cell block is exerted in a different way from the effect of deferoxamine.

Discussion

The present study showed that polyaminocarboxylates with various metal ion stability constants, including NTA, EDDA, EGTA, EDTA and DTPA, had the ability to overcome the two-cell block in development of ICR mouse embryos, whereas dipicolinic acid and deferoxamine, which are non-polyaminocarboxylate metal ion chelators, did not. EDTA caused maximum alleviation of the two-cell block at

$10 \mu\text{mol l}^{-1}$. At $10 \mu\text{mol l}^{-1}$, EDDA and DTPA, weak and strong chelators, respectively, compared with EDTA, had a similar ability to $10 \mu\text{mol EDTA l}^{-1}$ to overcome the two-cell block. Although NTA, like EDDA, is a weak chelator, and EGTA has a similar stability constant to EDTA, a higher concentration ($100 \mu\text{mol l}^{-1}$) of both NTA and EGTA was required to produce a significant increase in the proportion of embryos developing beyond the two-cell stage. This finding indicates that the ability of polyaminocarboxylates to overcome the two-cell block is not correlated with their potency to chelate transition metal ions. Moreover, it was found that deferoxamine at 1 and $10 \mu\text{mol l}^{-1}$ inhibited the first cleavage division of the embryos. As the stability constant of deferoxamine for iron is much greater than that of the other chelators examined in the present study, the adverse effect may be caused by chelation of an excess amount of iron in the extracellular pool (Nasr-Esfahani *et al.*, 1990). The adverse effect of deferoxamine was almost completely rescued by adding EDTA to deferoxamine-containing medium; furthermore, EDTA was able to promote the development of the embryos to the four-cell stage in deferoxamine-containing medium. As deferox-

Table 6. Effects of combined addition of deferoxamine and ethylenediaminetetraacetate (EDTA) on development of one-cell ICR mouse embryos *in vitro*

Additive	Number of embryos examined	Percentage of embryos developed to		
		Two-cell stage	Four-cell stage	Blastocyst
–	44	100 ^a	5 ^a	0 ^a
EDTA (10 $\mu\text{mol l}^{-1}$)	62	100 ^a	94 ^b	72 ^b
Deferoxamine (10 $\mu\text{mol l}^{-1}$)	49	53 ^b	0 ^a	0 ^a
EDTA (10 $\mu\text{mol l}^{-1}$) + deferoxamine (10 $\mu\text{mol l}^{-1}$)	50	98 ^a	83 ^b	0 ^a

^{ab}Values in the same column with different superscripts are significantly different.

amine and EDTA are both transition metal ion chelators, the results of this experiment indicate that the effect of EDTA on early embryos is exerted via a mechanism other than the chelation of iron.

In a study of one-cell ICR mouse embryos produced by *in vitro* fertilization, Hoshi and Toyoda (1985) reported that, unlike EDTA, EGTA (100 $\mu\text{mol l}^{-1}$) had no ability to promote development of the embryos *in vitro* beyond the two-cell stage, which is inconsistent with the results of the present study. It is possible that this discrepancy is the result of differences between the developmental potential of pronuclear stage one-cell embryos produced by fertilization *in vivo* and *in vitro*. Although the two-cell block of *in vitro* fertilized AKR/N mouse embryos cultured in phosphate-containing medium ($\geq 1 \mu\text{mol l}^{-1}$) was not alleviated by the addition of EDTA (Haraguchi *et al.*, 1996), the developmental arrest at the two-cell stage of *in vivo* fertilized AKR/N pronuclear stage embryos could be alleviated by the addition of 10 $\mu\text{mol l}^{-1}$ EDTA, even in phosphate-containing medium (T. Matsukawa, S. Ikeda and M. Yamada, unpublished).

Polyaminocarboxylates are generally believed to be cell membrane-impermeable chelators as they are negatively charged, which prevents their intracellular transport. Therefore, the various biological effects of polyaminocarboxylates are thought to be exerted extracellularly (Morimoto *et al.*, 1992; Chattopadhyay and Freake, 1998; Lefebvre *et al.*, 1998; Sciaudine *et al.*, 2000). If exogenous polyaminocarboxylates could be incorporated into early mouse embryos through endocytosis, they should be capable of acting intracellularly. However, early cleavage-stage mouse embryos lack effective endocytotic capacity (Nasr-Esfahani and Johnson, 1992). Moreover, when NTA and EDDA chelate metal ions, they form uncharged complexes that are able to penetrate the membrane and may also act inside the cell (Kachur *et al.*, 1998). However, as the effect of EDDA on alleviation of the two-cell block was similar to that of EDTA and DTPA, which are unable to penetrate the membrane of embryos, it is likely that the effect was predominantly the result of the extracellular action of polyaminocarboxylates. In preliminary experiments using microinjection of EDTA into either the cytoplasm or the perivitelline space of one-cell embryos, normal develop-

ment beyond the two-cell stage was observed only in embryos that received EDTA in the perivitelline space (Fissore *et al.*, 1989). The results of these experiments indicate that the alleviation effects of polyaminocarboxylates are exerted at or outside the vitelline membrane of the embryo. This contention is in agreement with the suggestion of Abramczuk *et al.* (1977) that EDTA may act on the cell surface of embryos. The results of the present study combined with these earlier findings indicate that the beneficial effects are not due to extracellular chelation of transition metal ions by polyaminocarboxylates and the consequent reduction of oxidative stress.

Treatment of early embryos at the cleavage stages with EDTA regulates their glycolytic metabolism, resulting in the suppression of the high glycolytic activity induced by abnormal utilization of glucose in culture (Gardner and Lane, 1993). The excess utilization of glucose by embryos at early cleavage stages has been implicated in the induction of embryonic arrest in culture (Barbehenn *et al.*, 1974; Chatot *et al.*, 1989; Brown and Whittingham, 1991, 1992; Martin and Leese, 1995), which appears to be mediated by the Crabtree effect, inhibition of respiration and oxidative phosphorylation by glucose (Crabtree, 1929; Seshagiri and Bavister, 1971). Moreover, it appears that the mechanism of suppression of the high glycolytic activity of early cleavage stage embryos by EDTA is due to the inhibition of cytosolic kinases involved in the glycolytic pathway (Lane and Gardner, 1997). Lane and Gardner (2001) reported that the two-cell block in CF1 mouse zygotes was overcome by treatment with Cibacron blue, an inhibitor of 3-phosphoglycerate kinase (3-PGK), which is a key enzyme in glycolysis, and that 3-PGK activity in F₁(C57BL/6 $\times$ CBA/Ca) hybrid mouse zygotes, which have no characteristic features of developmental block at the two-cell stage, was reduced by EDTA treatment. They also found that the intracellular content of magnesium ions, which are a co-factor of 3-PGK, was reduced by EDTA treatment in F₁ hybrid mouse embryos. Therefore, it was suggested that alleviation of the two-cell block in CF1 mouse zygotes by EDTA treatment is due to intracellular chelation of magnesium ions, causing a reduction in 3-PGK activity. However, in the present study, although NTA and EGTA

have much higher stability constants for magnesium ion than does EDDA (see Table 3), higher concentrations of NTA and EGTA than of EDDA were required to alleviate the two-cell block of ICR mouse embryos. Furthermore, Mg-EDTA, a non-magnesium ion chelator, at 10 or 100 $\mu\text{mol l}^{-1}$, alleviated the two-cell block in ICR mouse embryos to a similar extent as did EDTA.

The present study demonstrated that polyaminocarboxylates with different stability constants for transition metal ions have an ability to alleviate the two-cell block in ICR mouse zygotes; however, this ability was not necessarily correlated with the stability constants of the polyaminocarboxylates. Accordingly, although it remains to be clarified how EDTA and other polyaminocarboxylates alleviate the two-cell block in ICR mouse embryos, it seems likely that some common structural feature of polyaminocarboxylates, other than their ability to chelate metal ions, is required to promote embryonic development, especially beyond the second cell cycle division.

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References

- Abramczuk J, Solter D and Koprowski H (1977) The beneficial effects of EDTA on development of mouse one-cell embryos in chemically defined medium *Developmental Biology* **61** 378–383
- Asaumi A, Ogino T, Akiyama T, Kawabata T and Okada S (1996) Oxidative damages by iron–chelate complexes depend on the interaction with the target molecules *Biochemistry and Molecular Biology International* **39** 77–86
- Barbehenn EK, Wales RG and Lowry OH (1974) The explanation for the blockade of glycolysis in early mouse embryos *Proceedings National Academy of Sciences USA* **71** 1056–1060
- Brown JGG and Whittingham DG (1991) The roles of pyruvate, lactate and glucose during preimplantation development of embryos from F₁ hybrid mice *in vitro*. *Development* **112** 99–105
- Brown JGG and Whittingham DG (1992) The dynamic provision of different energy substrates improves development of one-cell random bred embryos *in vitro*. *Journal of Reproduction and Fertility* **95** 503–511
- Chatot CL, Ziomek CA, Bavister BD, Lewis JL and Torres I (1989) An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *Journal of Reproduction and Fertility* **86** 679–688
- Chattopadhyay S and Freake HC (1998) Zinc chelation enhances thyroid hormone induction of growth hormone mRNA in GH3 cells *Molecular and Cellular Endocrinology* **136** 151–157
- Crabtree HG (1929) Observations on the carbohydrate metabolism of tumors *Biochemical Journal* **23** 536–545
- Erbach GT, Lawitts JA, Papaioannou VE and Biggers JD (1994) Differential growth of the mouse preimplantation embryo in chemically defined media *Biology of Reproduction* **50** 1027–1033
- Fissore RA, Jackson KV and Kiessling AA (1989) Mouse zygote development in culture medium without protein in the presence of ethylenediaminetetraacetic acid *Biology of Reproduction* **41** 835–841
- Gardner DK and Lane M (1993) The 2-cell block in CF1 mouse embryos is associated with an increase in glycolysis and a decrease in tricarboxylic acid (TCA) cycle activity: alleviation of the 2-cell block is associated with the restoration of *in vivo* metabolic pathway activities *Biology of Reproduction* **48** Supplement 1 96
- Gardner DK and Lane M (1996) Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters *Human Reproduction* **11** 2703–2712
- Halliwell G and Gutteridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease *Biochemical Journal* **219** 1–14
- Haraguchi S, Naito K, Azuma S, Sato E, Nagahama Y, Yamashita M and Toyoda Y (1996) Effects of phosphate on *in vitro* 2-cell block of AKR/N mouse embryos based on changes in cdc2 kinase activity and phosphorylation states *Biology of Reproduction* **55** 598–603
- Hoshi M and Toyoda Y (1985) Effect of EDTA on the preimplantation development of mouse embryos *in vitro*. *Japanese Journal of Zootechnical Science* **56** 931–937
- Imamura S (1977) Application of complexan and its derivatives. In *EDTA – Chemistry of Complexan* pp 121–226 Ed. K Ueno. Nankodo Co Ltd, Tokyo
- Johnson MH and Nasr-Esfahani MH (1994) Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos *in vitro?* *BioEssays* **16** 31–38
- Kachur AV, Tuttle SW and Biaglow JE (1998) Autoxidation of ferrous ion complexes: a method for the generation of hydroxyl radicals *Radiation Research* **150** 475–482
- Lane M and Gardner DK (1997) EDTA stimulates development of cleavage stage mouse embryos by inhibiting the glycolytic enzyme 3-phosphoglycerate kinase *Biology of Reproduction* **57** Supplement 1 131
- Lane M and Gardner DK (2001) Inhibiting 3-phosphoglycerate kinase by EDTA stimulates the development of the cleavage stage mouse embryo *Molecular Reproduction and Development* **60** 233–240
- Larson AA and Kitto K (1999) Chelation of zinc in the extracellular area of the spinal cord, using ethylenediaminetetraacetic acid disodium-calcium salt or dipicolinic acid, inhibits the antinociceptive effect of capsaicin in adult mice *Journal of Pharmacology and Experimental Therapeutics* **288** 759–765
- Lawitts JA and Biggers JD (1992) Joint effects of sodium chloride, glutamine and glucose in mouse preimplantation embryo culture media *Molecular Reproduction and Development* **31** 189–194
- Lefebvre D, Beckers F, Ketelslegers JM and Thissen JP (1998) Zinc regulation of insulin-like growth factor-I (IGF-I), growth hormone receptor (GHR) and binding protein (GHBP) gene expression in rat cultured hepatocytes *Molecular and Cellular Endocrinology* **138** 127–136
- Legge M and Sellens MH (1991) Free radical scavengers ameliorate the 2-cell block in mouse embryo culture *Human Reproduction* **6** 867–871
- Martin KL and Leese HJ (1995) Role of glucose in mouse preimplantation embryo development *Molecular Reproduction and Development* **40** 436–443
- Morimoto S, Shiraishi T, Fukuo K, Koh E, Kitano S, Yasuda O, Tamatani M and Ogihara T (1992) EDTA induces differentiation and suppresses proliferation of promyelocytic leukemia cell line HL-60 – possible participation of zinc *Biochemistry International* **28** 313–321
- Nasr-Esfahani MH and Johnson MH (1992) How does transferrin overcome the *in vitro* block to development of the mouse preimplantation embryo? *Journal of Reproduction and Fertility* **96** 41–48
- Nasr-Esfahani M, Johnson MH and Aitken RJ (1990) The effect of iron and iron chelators on the *in vitro* block to development of the mouse preimplantation embryo: BAT6 a new medium for improved culture of mouse embryos *in vitro*. *Human Reproduction* **5** 997–1003
- Noda Y, Matsumoto H, Umaoka Y, Tatsumi K, Kishi J and Mori T (1991) Involvement of superoxide radicals in the mouse two-cell block *Molecular Reproduction and Development* **28** 356–360
- Okada S (1996) Iron-induced tissue damage and cancer. The role of reactive oxygen species-free radicals *Pathology International* **46** 311–332
- Payne SR, Munday R and Thompson JG (1992) Addition of superoxide dismutase and catalase does not necessarily overcome developmental retardation of one-cell mouse embryos during *in vitro* culture *Reproduction, Fertility and Development* **4** 167–174
- Sakaguchi S and Ueno K (1967) Appendix. In *Metal Chelate (III)* pp 1–78. Nankodo Co Ltd, Tokyo
- Sciaudone MP, Chattopadhyay S and Freake HC (2000) Chelation of zinc amplifies induction of growth hormone mRNA levels in cultured rat pituitary tumor cells *Journal of Nutrition* **130** 158–163

- Seshagiri PB and Bavister BD** (1991) Glucose and phosphate inhibit respiration and oxidative metabolism in cultured hamster eight-cell embryos: evidence for the 'Crabtree effect' *Molecular Reproduction and Development* **30** 105–111
- Stadtman ER** (1993) Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions *Annual Review of Biochemistry* **62** 797–821
- Yamada M, Okigaki T and Awai M** (1987) Role of superoxide radicals in cytotoxic effects of Fe-NTA on cultured normal liver epithelial cells *Cell Structure and Function* **12** 407–420
- Yamaguchi M and Matsui R** (1989) Effect of dipicolinate, a chelator of zinc, on bone protein synthesis in tissue culture. The essential role of zinc *Biochemical Pharmacology* **38** 4485–4489
- Zhao F, Yang J and Schoneich C** (1996) Effects of polyaminocarboxylate metal chelators on iron-thiolate induced oxidation of methionine- and histidine-containing peptides *Pharmaceutical Research* **13** 931–938
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