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: NTA ≤ EDDA < EGTA < EDTA < 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.

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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: NTA ≥ EDDA > EDTA > 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
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 i.u. equine chorionic gonadotrophin (eCG) followed 48 h later by 5 i.u. 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 µmol 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 µmol l$^{-1}$ and 10–100 µmol 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 1. Dose effects of ethylenediaminetetraacetate (EDTA) on development of one-cell ICR mouse embryos in vitro

<table>
<thead>
<tr>
<th>EDTA (µmol l$^{-1}$)</th>
<th>Number of embryos examined</th>
<th>Percentage of embryos developed to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Two-cell stage</td>
</tr>
<tr>
<td>0.0</td>
<td>47</td>
<td>100$^a$</td>
</tr>
<tr>
<td>0.25</td>
<td>50</td>
<td>100$^a$</td>
</tr>
<tr>
<td>0.5</td>
<td>55</td>
<td>100$^a$</td>
</tr>
<tr>
<td>1.0</td>
<td>55</td>
<td>100$^a$</td>
</tr>
<tr>
<td>10</td>
<td>56</td>
<td>100$^a$</td>
</tr>
<tr>
<td>100</td>
<td>38</td>
<td>100$^a$</td>
</tr>
<tr>
<td>1000</td>
<td>53</td>
<td>100$^a$</td>
</tr>
</tbody>
</table>

$^a$-$^c$ Values in the same column with different superscripts are significantly different.

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constants than magnesium and calcium ions, respectively. Mg–EDTA and Ca–EDTA at 10 and 100 μmol l–1 had similar effects to 10 and 100 μmol EDTA l–1 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 (> 95%) treated with EDDA, EDTA or DTPA at 10 or 100 μmol 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 μmol 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 μmol 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 (> 95%). The rates of development to the blastocyst stage of embryos treated with NTA or EGTA at 100 μmol l–1 (74% and 48%, respectively) were also lower compared with those (> 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 μmol dipicolinic acid l–1, 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 μmol deferoxamine l–1, 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 2. Effects of Ca– and Mg–ethylenediaminetetraacetate (EDTA) on development of one-cell ICR mouse embryos in vitro

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration (μmol l–1)</th>
<th>Number of embryos examined</th>
<th>Percentage of embryos developed to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Two-cell stage</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>65</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ca–EDTA</td>
<td>10</td>
<td>37</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>40</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mg–EDTA</td>
<td>10</td>
<td>38</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>38</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values in the same column with different superscripts are significantly different.

### Table 3. Stability constants of chelators for transition metal ions

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Zn&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Cu&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Fe&lt;sup&gt;3+&lt;/sup&gt;</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.7</td>
<td>13.0</td>
<td>15.9</td>
<td>6.4</td>
<td>5.5</td>
</tr>
<tr>
<td>EDDA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.2</td>
<td>16.2</td>
<td>17.0</td>
<td>na</td>
<td>3.9</td>
</tr>
<tr>
<td>EGTA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.5</td>
<td>17.8</td>
<td>20.5</td>
<td>11.0</td>
<td>5.2</td>
</tr>
<tr>
<td>EDTA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.5</td>
<td>18.8</td>
<td>25.1</td>
<td>11.0</td>
<td>8.7</td>
</tr>
<tr>
<td>DTPA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.8</td>
<td>21.5</td>
<td>28.6</td>
<td>10.7</td>
<td>9.3</td>
</tr>
<tr>
<td>Dipicolinic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.4</td>
<td>9.1</td>
<td>na</td>
<td>4.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Deferoxamine&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.1</td>
<td>14.1</td>
<td>30.6</td>
<td>2.6</td>
<td>na</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from Dojindo Laboratories, Kumamoto, Japan.
<sup>b</sup> Data from Sakaguchi and Ueno (1967).
<sup>c</sup> Data from Imamura (1977).

DTPA: diethylenetriaminepentacetic acid; EDDA: ethylenediaminediacetic acid; EDTA: ethylenediaminetetraacetate; EGTA: ethyleneglycolbistetraacetate; NTA: nitrilotriacetic acid; na: not available.
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 mM (Table 6), almost the same proportion of embryos developed to the four-cell stage as when the embryos were treated with 10 mM EDTA 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 mM. At 10 mM, EDDA and DTPA, weak and strong chelators, respectively, compared with EDTA, had a similar ability to 10 mM EDTA 1 mM 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 mM) 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 mM 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-
amino 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 μmol 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 (≥ 1 μmol 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 μmol EDTA l⁻¹, 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 endocytic 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 development 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 × 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 cofactor 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

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

<table>
<thead>
<tr>
<th>Additive</th>
<th>Number of embryos examined</th>
<th>Percentage of embryos developed to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Two-cell stage</td>
</tr>
<tr>
<td>–</td>
<td>44</td>
<td>100ᵇ</td>
</tr>
<tr>
<td>EDTA (10 μmol l⁻¹)</td>
<td>62</td>
<td>100ᵇ</td>
</tr>
<tr>
<td>Deferoxamine (10 μmol l⁻¹)</td>
<td>49</td>
<td>53ᵇ</td>
</tr>
<tr>
<td>EDTA (10 μmol l⁻¹) + deferoxamine (10 μmol l⁻¹)</td>
<td>50</td>
<td>98ᵇ</td>
</tr>
</tbody>
</table>

ᵃᵇValues in the same column with different superscripts are significantly different.
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 μmol 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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