Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH

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The inclusion of specific amino acids in conventional culture media has been shown to enhance mammalian embryo development in vitro. Amino acids have been shown to confer their benefits to the preimplantation embryo in a number of different ways. However, their ability to buffer intracellular pH (pHᵢ) has not been investigated. Thus, the aim of this study was to determine if amino acids regulate pHᵢ in the mouse preimplantation embryo. pHᵢ was determined using carboxy-seminaphthorhodafuor-1 (SNARF-1) and confocal microscopy. Incubation with 5,5-dimethyl-2,4-oxazolidinedione (DMO), a non-metabolizable weak acid, resulted in a significant intracellular acidification in the zygote, 2-, 4- and 8–16-cell embryo. However, in the presence of groups of amino acids, the degree of acidification due to DMO was markedly reduced in the mouse embryo up to the 4-cell stage. Specifically, non-essential amino acids and glutamine had the greatest capacity to buffer pHᵢ in the early embryo. The ability of amino acids to buffer pHᵢ was not apparent from the 8–16-cell stage onwards. In contrast to the precompacted embryo, the morula did not undergo a significant decrease in pHᵢ until exposed to DMO concentrations ≥10 mM in the absence of amino acids. This may be due to the generation of a permeability seal during compaction, thus enabling the morula to regulate its own pHᵢ. This regulatory ability could either be reversed by causing the morula to decompact, or created by inducing premature compaction in the 8–16-cell embryo. Data presented in this study indicate that amino acids act as buffers of pHᵢ in the early embryo and play a key role in regulating cell physiology. Further evidence for this was provided by the result that only those embryos cultured in 30 mM DMO in the presence of non-essential amino acids and 1 mM glutamine did not block at the 2-cell stage, but grew on to develop into expanded blastocysts.

Key words: amino acids/compaction/confocal microscopy/intracellular pH/SNARF-1

Introduction

Intracellular pH (pHᵢ) is known to be a key regulator of numerous intracellular processes in somatic cells (Busa and Nuccitelli, 1984). Despite its fundamental physiological importance, there is a relative paucity of information regarding the pHᵢ of the mammalian preimplantation embryo. A recent study used the pH-sensitive probe carboxy-seminalphthorhodafuor-1 (SNARF-1) and confocal microscopy to determine the pHᵢ of the mouse embryo at different stages of preimplantation development (Edwards et al., 1998). It was found that pHᵢ was not affected by extracellular pH (pHₑ) within a physiological range (7.20–7.80), but that the presence of weak acids, such as lactate, in the medium did perturb both the pHᵢ and physiology of the early embryo. In contrast, compacted embryos were capable of regulating pHᵢ when challenged with an acid load.

Amino acids, in particular taurine, glycine, alanine, glutamate and aspartate, have been detected at very high concentrations in mammalian oviduct fluid and preimplantation embryos (Schultz et al., 1981; Miller and Schultz, 1987). The concentrations of these amino acids are far in excess of the metabolic requirements of the mammalian preimplantation embryo and are therefore indicative of some other role in embryo development. It has been demonstrated in a number of different mammalian species that the addition of amino acids to culture media markedly improves embryo development in vitro (mouse, Gardner and Lane, 1993a; hamster, Carney and Bavister, 1987; rat, Zhang and Armstrong, 1990; sheep, Gardner et al., 1994; cow, Takahashi and First, 1992; human, Gardner and Lane, 1997; Gardner, 1998). Specifically, it was established that non-essential amino acids increased the cleavage rate of the first three cell divisions and enhanced blastocoel development and hatching in the mouse embryo (Gardner and Lane, 1993a; Lane and Gardner, 1997a,b). In contrast, essential amino acids which did not stimulate the rate of cleavage until after the 8-cell stage, stimulated the development of the inner cell mass (Lane and Gardner, 1997b). Amino acids have been shown to confer their benefit to the preimplantation embryo in a number of different ways by acting as metabolic substrates (Kane and Foote, 1970; Rieger and Guay, 1988; Chatot et al., 1990), anabolic precursors for protein synthesis (Monesi and Salfi, 1967), osmolytes (Van Winkle et al., 1990a; Lawitts and Biggers, 1992; Biggers et al., 1993; Dawson and Bultz, 1997; Dumoulin et al., 1997), chelators of heavy metal ions (Van Winkle and Campione, 1982) and as regulators of metabolic function (Gardner and Lane, 1993b; Gardner, 1998). However, as amino acids exist as zwitterions at physiological pH, it is conceivable that they can also act as buffers of pHᵢ (Bavister and McKiernan, 1993; Gardner and Lane, 1997). This potential buffering capacity is of considerable importance when considering the fact that the early embryo appears to lack mechanisms to alleviate an acid load (Bultz et al., 1990) and that lactate, a weak acid and...
an essential component of embryo culture media, induces intracellular acidification and metabolic perturbations in the early embryo (Edwards et al., 1998). The ability of amino acids to buffer pH\textsubscript{i} in the mammalian preimplantation embryo has not been investigated.

Thus, the aims of this study were to determine whether amino acids could buffer pH\textsubscript{i} in the mouse embryo at different stages of preimplantation development, and to investigate the physiological implication of this ability on embryo development in vitro.

Materials and methods

Media and solutions

A modified mouse tubal fluid medium (mMTF; Gardner and Lane, 1993a) was used for embryo culture which had the following composition: 98.40 mM NaCl, 4.79 mM KCl, 1.19 mM KH\textsubscript{2}PO\textsubscript{4}, 1.71 mM CaCl\textsubscript{2}, 2H\textsubscript{2}O, 1.19 mM MgSO\textsubscript{4}, 7H\textsubscript{2}O, 25 mM NaHCO\textsubscript{3}, 0.37 mM sodium pyruvate, 4.79 mM l-/d-sodium lactate, 3.40 mM glucose, 0.06 g/l penicillin, 0.05 g/l streptomycin, 0.01 g/l phenol red and 4 g/l bovine serum albumin (BSA; Gibco-BRL). Life Technologies Ltd, Glen Waverley, Victoria, Australia). In media used for all embryo collections and manipulations, 20 mM NaHCO\textsubscript{3} was replaced with 20 mM HEPES (H-mMTF). All salts were of AnalaR grade (BDH, Poole, Dorset, UK): glucose, pyruvate, lactate, antibiotics, HEPES and phenol red were embryo-tested (Sigma Chemical Co., St Louis, MO, USA). Media were supplemented with non-essential amino acids and 1 mM glutamine (NEGLN; Sigma) or essential amino acids either with 1 mM glutamine (ESSGLN) or without (ESS). Non-essential and essential amino acids were purchased from ICN Biomedicals (Seven Hills, NSW, Australia). 5,5-Dimethyl-2,4-oxazol-idinedione (DMO; Sigma) was present in all culture and handling media at a concentration of either 0, 1, 5, 10, or 30 mM. The pH\textsubscript{o} of all media was maintained at 7.4.

H-mMTF, from which both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} ions were omitted, was used to induce decompaction in embryos collected at the morula stage. The decompaction medium was supplemented with 5 µg/ml cytochalasin B (Sigma) which was prepared as a 5 mg/ml stock solution in dimethylsulphoxide (DMSO; Sigma) and stored at −20°C (Sutherland and Calarco-Gillam, 1983). Wheat germ agglutinin (WGA; Sigma) used to induce compaction was prepared as a 10 mg/ml stock solution in the appropriate culture medium (Johnson, 1986). SNARF-1 (Molecular Probes Inc., Eugene, OR, USA) was prepared as a 1 mM stock solution dissolved in dry DMSO and stored at −20°C. Solutions and ionophores for the in-vivo calibration of pH\textsubscript{i} differences that may have occurred, images were taken from the equatorial plane of the embryo. Furthermore, apertures were set in each emission pathway to ensure that the images collected were of equal thickness. At the conclusion of each experiment, an in-vivo calibration to alter the ratio values recorded to pH\textsubscript{i} was performed as described by Edwards et al. (1998). Briefly, embryos were incubated for 10 min in a high K\textsuperscript{+} calibration solution supplemented with nigericin (10 µg/ml; Sigma) and valinomycin (5 µg/ml; Sigma). Embryos were then exposed to calibration solutions of varying pH (6.8–7.4) and the resulting pH\textsubscript{i} measured.

Experiment 1. Effect of DMO on pH\textsubscript{i} of the mouse preimplantation embryo in the presence or absence of amino acids

Embryos were collected from the female reproductive tract at either the zygote, 2-, 4-, 8–16-cell or morula stage in either H-mMTF, H-NEGLN, H-ESSGLN or H-ESS. As glutamine has been shown to be an important essential amino acid in mouse embryo development (Gardner and Lane, 1993a; Lane and Gardner, 1997a), the effect of essential amino acids with or without glutamine was assessed. Zygotes were denuded using hyaluronidase as described. Embryos were loaded with SNARF-1 and baseline pH\textsubscript{i} determined. Embryos were then incubated in handling medium containing DMO at a concentration of 1, 5, 10 or 30 mM. pH\textsubscript{i} was measured after a 10 min incubation. Throughout the experimental time period embryos were maintained under the same conditions in which they were collected (H-mMTF; H-NEGLN, H-ESSGLN or H-ESS). The pH\textsubscript{o} of all media was maintained at 7.4.

Experiment 2. Effect of DMO on embryo development in vitro

Zygotes were collected as described and cultured in groups of 10 in 20 µl drops of culture medium in 35-mm Primaria Petri dishes (Falcon; Becton-Dickinson, Victoria, Australia) under a layer of mineral oil (Sigma) at 37°C in 5% CO\textsubscript{2} in air. Embryos were cultured in increasing concentrations of DMO in either mMTF, NEGLN, ESSGLN or ESS. On day 3 of culture, embryos were transferred to fresh drops of like culture media. Blastocyst development was assessed at 96 h of culture.

Experiment 3. Role of compaction and amino acids on pH\textsubscript{i} regulation in the mouse preimplantation embryo

Decompaction of morulae

Morulae, flushed from the reproductive tract 68 h post HCG, were decompacted by incubation in Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free H-mMTF supplemented with 5 µg/ml cytochalasin B (Sutherland and Calarco-Gillam, 1983) for 10 min. Embryos were then washed in Mg\textsuperscript{2+}-free H-mMTF, supplemented with 5 µg/ml cytochalasin B, and the baseline pH\textsubscript{i} determined. Decom pared embryos were subsequently exposed to either 1, 5, 10 or 30 mM DMO for 10 min and the resulting pH\textsubscript{i} recorded. The pH\textsubscript{o} of all media was maintained at 7.4. The effect of amino acids was not determined in these experiments due to their...
apparent loss of buffering capacity after the 8–16-cell stage (Figure 1d and e).

Compaction of 8–16-cell embryos

Early 8–16-cell embryos were flushed from the reproductive tract 63 h post HCG in H-mMTF or H-ESSGLN. Compaction was induced by incubation with 50 µg/ml WGA (Johnson, 1986) for at least 90 min or until no individual blastomeres were visible. Embryos were then washed free of WGA and their pH measured before and after exposure to DMO at either 1, 5, 10 or 30 mM in the presence or absence of essential amino acids as described. Due to their beneficial effect on embryo development after the 8-cell stage, essential amino acids were included in order to assess their importance in the process of compaction.

Statistical analysis

Values are reported as mean ± SEM. Mean embryo pH was determined before and after exposure to increasing concentrations of DMO. Differences between the control and treatment groups were analysed using the Mann–Whitney test. Blastocyst formation was expressed as a percentage of total embryos cultured. Differences between embryo culture treatment groups were determined after analysis of variance (ANOVA) followed by the Bonferroni multiple comparison procedure (Ludbrook, 1991).

Results

Experiment 1. Effect of DMO on pH of the mouse preimplantation embryo in the presence or absence of amino acids

The baseline pH of zygotes collected in H-mMTF was 7.18 ± 0.01 (n = 80). Following a 10-min incubation in the absence of DMO, it was observed that there was a significant decrease in pH at all concentrations used (P < 0.05; Figure 1a). Zygotes collected in H-NEGLN had a baseline pH of 7.21 ± 0.01, significantly higher than those collected in H-mMTF (7.18 ± 0.01; P < 0.05). Zygotes collected in H-ESSGLN and H-ESS had a baseline pH of 7.20 ± 0.01 and 7.19 ± 0.01, respectively. In the presence of amino acids, DMO did not induce a significant intracellular acidification within the zygote until added at concentrations of 5 mM or greater (P < 0.01; Figure 1a). It was apparent that the magnitude of intracellular acidification due to DMO was not as large in the presence of amino acids as in their absence (Figure 1a). Indeed, in 5 mM DMO, the pH of embryos maintained in H-NEGLN and H-ESSGLN and H-ESS were 7.17 ± 0.01, 7.13 ± 0.01 and 7.12 ± 0.01, respectively. It was observed at all DMO concentrations used that zygotes held in H-NEGLN had a significantly higher pH than those in the remaining treatment groups (P < 0.01).

Similarly, the 2-cell embryo underwent significant intracellular acidification in response to DMO (Figure 1b). Both the 2- and 4-cell stages responded to amino acids in a manner similar to that seen in the zygote with the following exceptions. Firstly, the pH of the 2-cell embryo did not undergo a significant fall in pH until exposed to a concentration of 5 mM DMO when maintained in H-MTF. 10 mM DMO in H-ESSGLN or H-ESS, and 30 mM DMO in H-NEGLN (P < 0.01; Figure 1b). The pH of 2-cell embryos maintained in H-NEGLN was significantly higher than those in H-MTF, H-ESSGLN or H-ESS at 10 and 30 mM DMO (P < 0.01). Secondly, non-essential and essential amino acids supplemented with glutamine appeared to have a similar buffering capacity in the 4-cell embryo (Figure 1c). Furthermore, at 5 and 10 mM DMO, the pH of embryos in H-NEGLN, H-ESSGLN and H-ESS were significantly greater than those maintained in H-MTF (P < 0.05). There was minimal (3%) variation in pH between the individual blastomeres of an embryo.

The baseline pH of 8–16-cell embryos ranged from 7.22 to 7.23 in the presence or absence of amino acids (Figure 1d). In all treatment groups the pH of the 8–16-cell embryo became significantly more acidic following a 10-min incubation in DMO (concentrations 1–30 mM; P < 0.01). It was interesting to note that this apparent change in the amino acids’ buffering ability was the same in zygotes and 8–16-cell embryos exposed to an alkaline load (data not shown).

The mean baseline pH of morulae collected in H-mMTF was 7.21 ± 0.01 (n = 80) and did not change significantly until exposed to a DMO concentration of 10 mM (7.18 ± 0.01, P < 0.05; Figure 1e). It was apparent that the intracellular acidification caused by DMO was not as dramatic in the compacted embryo as was observed in the precompacted embryo. Amino acids did not appear to affect pH when the embryo was exposed to DMO, as no significant differences between treatment groups were identified.

Experiment 2. Effect of DMO on embryo development in vitro

84% of embryos cultured from the zygote were expanded blastocysts after 96 h of culture in mMTF, 88% in NEGLN, 83% in ESSGLN, and 90% in ESS (Table I). Upon culture with increasing concentrations of DMO, development to the blastocyst stage was significantly reduced in the presence of 30 mM DMO (P < 0.01). Embryos cultured in mMTF, ESSGLN and ESS did not reach the blastocyst stage, but instead became blocked at the 2-cell stage. In contrast, when cultured in NEGLN and exposed to 30 mM DMO, 58% of zygotes reached the blastocyst stage.

Experiment 3. Role of compaction and amino acids on pH regulation in the mouse preimplantation embryo

Decomposition of morulae

With the removal of Ca2+ and Mg2+ ions from, and addition of cytochalasin B to, the handling medium it was possible to cause morulae to decompact. This procedure did not appear to be detrimental to the embryo, as the pH in decompacted embryos (7.21 ± 0.01, n = 80) did not differ from that measured in untreated embryos (7.21 ± 0.01). Furthermore, upon replacing the decompacted embryos in conventional medium containing both Ca2+ and Mg2+ ions, recompaction occurred within 10 min. Incubation in ≥5 mM DMO resulted in intracellular acidification in the decompacted embryo (P < 0.01; Figure 2).

Compaction of 8–16-cell embryos

The 8–16-cell embryos exposed to WGA for 90 min had a morphology resembling that of compacted morulae. Control
Figure 1. Effect of increasing 5,5-dimethyl-2,4-oxazolidinedione (DMO) concentration (mM) on the intracellular pH (pH$_i$) of the mouse (a) zygote, (b) 2-cell, (c) 4-cell, (d) 8–16-cell and (e) morula following a 10-min incubation. Embryos were treated in either modified mouse tubal fluid medium buffered with HEPES (H-mMTF; ○), H-mMTF supplemented with non-essential amino acids and glutamine (H-NEGLN; ○), H-mMTF supplemented with essential amino acids and glutamine (H-ESSGLN; ▲) or H-mMTF supplemented with essential amino acids (H-ESS; △). Controls are 0 mM DMO H-mMTF, -NEGLN, -ESSGLN or -ESS, n = 80. Values are mean ± SEM. n = 20 embryos for each treatment group, from at least three replicates. *P < 0.05; **P < 0.01 (significantly different from like controls); a–s like pairs significantly different (P < 0.05).
Amino acids buffer intracellular pH in the mouse embryo

Figure 2. Effect of increasing 5,5-dimethyl-2,4-oxazol-idinedione (DMO) concentration (mM) on the intracellular pH (pHi) of decompacted mouse morulae following a 10-min incubation. Embryos were manipulated in modified mouse tubal fluid medium buffered with HEPES (H-mMTF; ●). Control was 0 mM DMO H-mMTF, n = 80. Values are mean ± SEM. n = 20 embryos, from at least three replicates. **P < 0.01 versus control.

Figure 3. Effect of increasing 5,5-dimethyl-2,4-oxazol-idinedione (DMO) concentration (mM) on the intracellular pH (pHi) of 8–16-cell mouse embryos following a 10-min incubation. Embryos were induced to compact by incubation with wheat germ agglutinin in either modified mouse tubal fluid medium buffered with HEPES (H-mMTF; ●) or H-mMTF supplemented with essential amino acids and glutamine (-ESSGLN, △). Controls are 0 mM DMO H-mMTF or -ESSGLN, n = 80. Values are mean ± SEM. n = 20 embryos, from at least three replicates. **P < 0.01 versus comparable control.

Table 1. Effect of increasing 5,5-dimethyl-2,4-oxazol-idinedione (DMO) concentration (mM) on blastocyst development (%) of mouse zygotes cultured in mMTF or mMTF supplemented with either non-essential amino acids and 1 mM glutamine (NEGLN) or essential amino acids with (ESSGLN) or without 1 mM glutamine (ESS) for 96 h.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>DMO (mM)</th>
<th>mMTF</th>
<th>NEGLN</th>
<th>ESSGLN</th>
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<tr>
<td>0 (Control)</td>
<td>84</td>
<td>88</td>
<td>83</td>
<td>90</td>
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<td>1</td>
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<td>10</td>
<td>76</td>
<td>85</td>
<td>68</td>
<td>67b</td>
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<tr>
<td>30</td>
<td>0c</td>
<td>58hde</td>
<td>0ad</td>
<td>0c</td>
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</tbody>
</table>

mMTF, mouse tubal fluid medium. n = at least 60 embryos cultured per treatment group, six replicates.

aBlocked at 2-cell stage.

bSignificantly different to comparable control within columns, P < 0.01.

c,d,e Like pairs significantly different, P < 0.01.

Discussion

Amino acids have been shown to be of significant benefit to the in vitro development of the preimplantation embryo (Carney and Bavister, 1987; Takahashi and First, 1992; Gardner and Lane, 1993a; Gardner et al., 1994) and a number of functional roles have been demonstrated. However, an important function of amino acids, which has received little investigation, is their potential ability to buffer pH. At physiological pH, amino acids exist as zwitterions and as such are able to chelate or release H⁺ ions. Thus, amino acids may play a role in pH regulation and ion balance within the preimplantation embryo. It is of interest to note that controversy surrounds the ability of the mouse 2-cell embryo to extrude H⁺ ions (Baltz et al., 1990; Gibb et al., 1997). It has been demonstrated that weak acids such as lactate, present in embryo culture media, induce both intracellular acidification and metabolic perturbations in the early embryo (Edwards et al., 1998). Amino acids have been shown to counteract the perturbing effect of lactate on pH in the zygote (L.J.Edwards and D.K.Gardner, unpublished observations). Thus, the apparent ability of amino acids to buffer pH would in part explain their beneficial effects on embryo development in vitro.

It was the aim of this study to investigate the capacity of amino acids to buffer pH in the preimplantation mouse embryo. pH was measured using the ratiometric dye, SNARF-1, in conjunction with confocal microscopy. Control values for the pH of the mouse preimplantation mouse embryo determined in this study were consistent with those recorded previously using similar techniques (Edwards et al., 1998). To determine the ability of amino acids to buffer pH, mouse embryos were challenged with DMO, a non-metabolizable weak acid, and the pH measured. Embryos were exposed to DMO in amino acid-free medium or in the presence of different groups of amino acids. It was observed that amino acids did have the ability to buffer pH against an acid load in the early embryo from the zygote to the 4-cell stage. Of the three amino acid groups studied, non-essential amino acids and glutamine had the greatest capacity to buffer pH against an acid load in the early embryo, particularly at the zygote stage. Interestingly, this group of amino acids was shown to be of the greatest benefit to the early embryo during development in vitro (Gardner and Lane, 1993a,b; Lane and Gardner, 1997a,b). Of the essential amino acids, glutamine proved to be of great significance.
importance, as the simple inclusion of this amino acid to ESS resulted in a greater buffering capacity than in its absence. In contrast to the pH reduction evident in the zygote, DMO induced a decrease in pH in the 8–16-cell embryo of the same magnitude as the control, regardless of the presence of amino acids. It appeared that with development to the 8–16-cell stage, the capacity of amino acids to regulate pH was lost.

Transport systems have been shown to exist in the plasma membrane of the mammalian embryo with the specific role of moving amino acids across the bilayer. The mechanisms of action of these amino acid transporters, their sensitivity and interactions have been reviewed by Kaye (1986) and Van Winkle (1988). These amino acid transport systems have the capacity to shuttle H⁺ ions into and out of the cell, and therefore the capacity to regulate pH and ionic balance within the embryo. Indeed, amino acid transport has also been called ion transport (Christensen, 1984). It was observed that the early mouse embryo was capable of utilizing the zwitterionic nature of amino acids to buffer pH in the face of an acid load, but was unable to do so after the 8-cell stage. The major facilitative amino acid carrier system present in the early embryo has been characterized as that of the Na⁺/Cl⁻-dependent system (Kaye et al., 1982; Hobbs and Kaye, 1986; Van Winkle et al., 1988; Lewis and Kaye, 1992). Therefore, the predominant mechanism employed to transport amino acids into the early embryo, that of the L system, is not coupled to Na⁺ ions (Lewis and Kaye, 1992). As a result, amino acids would be transported into the embryo as zwitterions and be free to chelate excess H⁺ ions inside the cell, thus buffering the pH. Amino acids may also be counter-exchanged for protons as they enter the embryo, and so counteract an acid load. It is interesting to note that the influx of amino acids and the establishment of a concentration gradient makes it possible for the Na⁺/Cl⁻-dependent L system to reverse its direction, causing the export of amino acids (Kaye et al., 1982; Christensen, 1984; Kaye, 1986). Bavister and McKiernan (1993) proposed that the efflux of amino acids from the embryo would result in the loss of H⁺ ions and in cytoplasmic alkalization. Indeed, the efflux of amino acids from the embryo did result in an increase in pH (L.J.Edwards and D.K.Gardner, unpublished observations), indicating that these mechanisms are in operation.

Borland and Tasca (1974) demonstrated that in the late 4-cell mouse embryo the transport of methionine and leucine was Na⁺/Cl⁻-independent. In contrast, the morula exhibited partial dependency to Na⁺ in the transport of these amino acids (Borland and Tasca, 1975), whereas the early blastocyst was totally Na⁺/Cl⁻-dependent (Borland and Tasca, 1974, 1975). Furthermore, Chatot et al. (1990) observed that glutamine transport, coupled with Na⁺, dramatically increased after the 8-cell stage. The greatest expression of Na⁺/Cl⁻-dependent amino acid transport (B⁺Cl⁻) mechanisms was observed after compaction and with the development of the blastocyst (DiZio and Tasca, 1977; Hobbs and Kaye, 1986; Van Winkle and Campione, 1990; Van Winkle et al., 1990b, 1991). As the embryo develops, carrier-mediated amino acid transport systems change from being predominantly Na⁺/Cl⁻-dependent to Na⁺/Cl⁻-independent, and may reflect the changing Na⁺ concentration within the oviduct fluid (Van Winkle et al., 1985). It is feasible that this change in amino acid transport systems utilized by the preimplantation embryo is also linked to the observed change in apparent ability of amino acids to buffer pH in the mouse preimplantation embryo from the 8–16-cell stage (Figure 1d). Furthermore, it was apparent that non-essential amino acids and glutamine had a greater buffering capacity than the other amino acid groups in the zygote and 2-cell embryo (Figure 1a and b). However, in the 4-cell embryo, non-essential amino acids and glutamine buffered pH to the same magnitude as essential amino acids and glutamine (Figure 1c). This apparent change in buffering capacity may reflect the initiation of the documented change in amino acid transport systems. It is interesting to note that Kaye et al. (1982) reported that Na⁺-dependency appeared in the embryo just before the rapid exchange capacity of the L system was lost at the 8–16-cell stage. Thus, the apparent inability of amino acids to buffer pH from the 8–16-cell stage was unlikely to be due to a loss of their zwitterionic properties, but rather reflected a change in the predominant amino acid transport system in operation. The mechanisms of Na⁺-dependent amino acid transport in the embryo are not known, although K⁺ ions have also been found to be linked to the movement of amino acids into the mouse embryo around the time of compaction (DiZio and Tasca, 1977). However, the stoichiometry of a Na⁺-dependent glutamate transporter has been characterized in glial cells (Bouvier et al., 1992) and in the Xenopus oocyte (Mackenzie et al., 1994). Glutamate was co-transported into the cell in its anionic form with Na⁺ ions. As an anion, glutamate would not be able to function as a zwitterion upon entering the cell, and would therefore be incapable of chelating excess H⁺ ions. In order to maintain electroneutrality, K⁺ and either OH⁻ or HCO₃⁻ ions were transported out of the cell. Therefore, with the loss of these ions, the cell can no longer alleviate an acid load; indeed, their efflux causes intracellular acidification. The existence of this transporter in the 8–16-cell embryo would explain the loss of amino acid buffering capacity.

This study has demonstrated that amino acids buffer pH within the early preimplantation embryo. In order to determine the physiological relevance of this observation, zygotes were cultured in increasing concentrations of DMO in either mMTF or in mMTF supplemented with either non-essential amino acids and glutamine, essential amino acids and glutamine, or essential amino acids. Blastocyst development was unaffected by the presence of DMO up to a concentration of 10 mM (Table I). However, development rates were lower in 10 mM DMO than in the controls, particularly in media supplemented with essential amino acids without glutamine (P < 0.01). A further increase in DMO concentration to 30 mM resulted in embryos blocking at the 2-cell stage in all media, except that supplemented with NEGLN. However, development was normal when embryos were cultured from the 2-cell stage in 30 mM DMO in the absence of amino acids (L.J. Edwards and D.K. Gardner, unpublished observations). Although it is not possible to discount the osmoprotective role that amino acids play, data presented in this study suggest that arrest at the 2-cell stage is a pH-sensitive phenomenon and that for continued
Amino acids buffer intracellular pH in the mouse embryo

development of the zygote to the blastocyst to occur, the pH\textsubscript{i} must not fall below 7.1. The presence of non-essential amino acids and glutamine allowed the embryo to have a greater capacity to buffer protons and hence pH\textsubscript{i} did not fall below this critical point. In 30 mM DMO only those zygotes cultured in NEGLN were capable of developing to the blastocyst stage. These data further support the importance of amino acids and their inclusion in embryo culture media. This is particularly true in light of the recent work of Dale et al. (1998), in which it was found that the human oocyte and embryo could not regulate against an acid load. Amino acids in the culture medium could therefore protect the human embryo against acid-induced trauma.

It was demonstrated that the morula was capable of maintaining baseline pH\textsubscript{i} and normal physiology when challenged with lactic acid (Edwards et al., 1998). Similarly, incubation with increasing concentrations of DMO did not affect pH\textsubscript{i} in the morula to the same extent as in the early embryo (Figure 1d). As compaction proceeds, zonular tight junctions form between the apical surfaces of external blastomeres, as indicated by transmission electron microscopy, freeze fracture and lanthanum exclusion techniques (Ducibella and Anderson, 1975; Ducibella et al., 1975). The formation of a permeability seal thus initiates the generation of the first transporting epithelium in the blastocyst (Biggers et al., 1987). By definition, epithelial cells are polarized and joined by tight junctions which enhance their ability to control the vectorial movement of both electrolytes and non-electrolytes (Lewis, 1996). It is possible that these cells are also capable of regulating the movement of H\textsuperscript{+} ions in the embryo. Observations in this study have shown that the compacted embryo is indeed capable of maintaining pH\textsubscript{i} even in the presence of the weak acid DMO (Figure 1d). However, when connections between adherent blastomeres were disrupted by the removal of Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, the morula lost its ability to regulate its internal environment, even in the presence of essential amino acids (Figure 2), a behaviour evident in the 8–16-cell embryo. Similarly, decompacted embryos when placed in media containing both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} were capable of recompaction within 10 min. Conversely, with the use of WGA, which acts by increasing intracellular apposition and adhesion between blastomeres (Johnson, 1986), it was possible to induce compaction-like events in the 8-cell embryo. With the disruption of cell polarization during decompaction it appears that the membrane-transport properties of the morula are lost. However, an 8–16-cell embryo, which has no apparent means of alleviating an acid load (Figure 1d), when treated with WGA, behaved like a morula (Figures 1e and 3). This behaviour is highly indicative that pH\textsubscript{i} regulation after compaction is due to the formation of a physical barrier rather than to the existence of membrane transporters. Thus, it appears that the tight association of adjacent blastomeres is of paramount importance to the ability of the morula to regulate its own pH\textsubscript{i}.

In conclusion, amino acids were found to buffer pH\textsubscript{i} in the mouse embryo up to the 4-cell stage. Of the amino acid groups studied, non-essential amino acids and glutamine had the greatest capacity to buffer pH\textsubscript{i} and were associated with embryo development in culture in the presence of an acid load (DMO). At the 8–16-cell stage, the ability of amino acids to regulate pH\textsubscript{i} was lost. This switch in buffering capacity may be associated with a change in the dynamics of the amino acid transport systems in operation. Due to the formation of a permeability seal during compaction, the morula was able to control its own pH\textsubscript{i} and be independent of its external environment. Such data are consistent with the concept that the morula is more robust than the cleavage-stage embryo.

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References


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