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In partial fulfilment of the requirements
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Department of Cellular and Molecular Medicine
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ABSTRACT

It has previously been shown that high osmolarity is detrimental to cleavage-stage mouse embryo development in vitro, and that the presence of several organic osmolytes can provide protection against the detrimental effects of raised osmolarity. Whether the same is true for post-compaction stage embryos is unknown. In the present work, it was found that mouse post-compaction stage embryo development was inhibited by raised osmolarity. However, inhibition of embryo development from the 8-cell to the blastocyst stage occurred only at much higher osmolarities than that which inhibited development from the 1-cell stage. Glutamine, glycine, L-alanine and β-alanine, which have been proven to function as organic osmolytes providing protection against increased osmolarity in cleavage-stage embryos (during the 1-cell through 4-cell stages), also protected post-compaction stage embryos from the detrimental effects of high osmolarity. Two other organic osmolytes, betaine and proline, which are effective in pre-compaction embryos, were not effective in providing protection against raised osmolarity in embryos developing in vitro from the 8-cell stage, nor were myo-inositol and taurine, which have been shown to be ineffective in cleavage-stage embryos. In addition, cleavage-stage embryos and post-compaction stage embryos were found to use different transport mechanisms to accumulate the four organic osmolytes that provided them with osmoprotection. When assessed in morulae, the amino acid transport system β was found to be responsible for β-alanine transport, while transport system B^{0+} mediated transport of glutamine, glycine and L-alanine. Glutamine, glycine, L-alanine and β-alanine supported post-compaction stage embryo development, higher embryo cell number in blastocysts, and greater embryo volume at higher osmolarities. The four compounds identified do not

share metabolic pathways or other such properties in common, and thus it is likely that post-compaction mouse embryos utilize them, in large part, as organic osmolytes.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BCH	2-amino-endo-bycyclo[2.2.1]heptane-2-carboxylic acid
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
Fig.	Figure
FSH	Follicle stimulation hormone
GLYT1	Glycine transporter 1
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
Hoechst 33258	Bisbenzimide
ICM	Inner cell mass
KFHM	Hepes-KSOM
KSOM	K^+ supplemented optimized medium
LH	Luteinizing hormone
nL	Nano litre
NS	Not significant
PB	Polar body
PMSG	Pregnant mare's serum gonadotropin
PN	Pronuclei
PVA	Polyvinyl alcohol
ORG	bis(4-fluorophenyl) methylenepiperidineacetic acid, lithium salt
RVI	Regulatory volume increase
SEM	Standard error of the mean
TE	Trophectoderm
ZP	Zona pellucida

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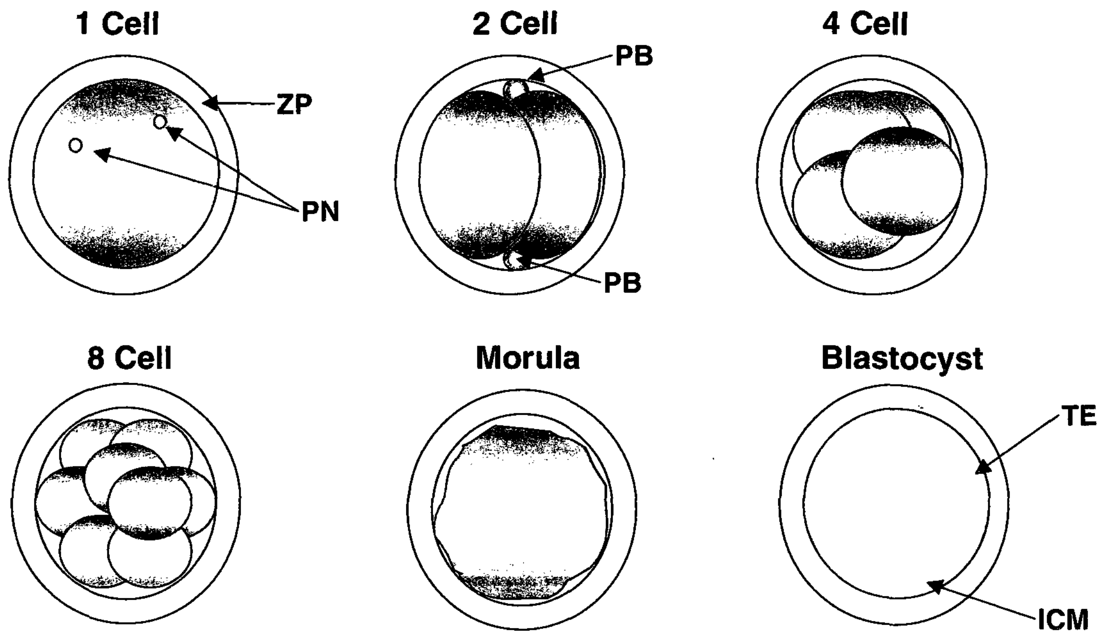
INTRODUCTION

Preimplantation Embryo Development

An embryo begins at the point of fertilization when the head of the sperm fuses with the membrane of the egg, incorporating the paternal genetic material of the sperm nucleus into the egg, which contains the maternal genetic material (Wassarman, 1987). Soon after sperm-egg fusion, maternal and paternal haploid pronuclei, each containing the haploid genetic material from one parent, form and migrate to the centre of the egg (Wassarman, 1987). The presence of the two pronuclei marks the formation of a one-cell zygote (Longo, 1973). The zygote then undergoes successive mitotic divisions or cleavages through about three cell cycles before beginning to differentiate into different cell lineages just before implanting in the uterus (Calarco and Brown, 1969; Brinster, 1974). Prior to implantation, the embryo is known as a preimplantation embryo. Preimplantation embryos may be classified into two stages, the cleavage-stage embryo and the post-compaction stage embryo, whose features are described in detail below.

Figure 1: Preimplantation Embryo Development

After fertilization, when the sperm cell penetrates the oocyte, a one-cell zygote is formed in which two pronuclei (PN) are visible. After 24 hours the first mitotic division occurs forming a two-cell embryo. Within 48 hours after fertilization the embryo has undergone a second mitotic division creating a four-cell embryo. On day three post-fertilization the embryo cleaves to form an eight-cell embryo. By day 3.5 the embryo has undergone successive cleavages forming a round sphere of compact cells called a morula. At approximately 4.5 days post-fertilization, the dense cluster of cells has developed into a hollow ball of cells forming two cell lineages, the inner cell mass (ICM) and the trophectoderm (TE) at this stage the embryo is called a blastocyst. Timing given here is for the mouse, and varies somewhat between mammalian species.



Cleavage-Stage Embryogenesis

The 1-cell zygote to the early 8-cell embryo is considered a cleavage-stage embryo (Hogan *et al.,* 1994). The zygote or 1-cell embryo stage persists for about 24 hours in mammals. At the end of this first embryonic cell cycle, the zygote enters the first mitotic metaphase, in which the chromosomes from the female and male pronuclei align on a common spindle (Fleming and Johnson, 1988). Completion of mitosis gives rise to a 2-cell embryo in which each cell contains the entire new diploid genome (Cooper, 2000). The 2-cell embryo is formed approximately 24 hours post-fertilization. Within 48 hours after fertilization the embryo undergoes its second mitotic division and a 4-cell embryo is formed. On day three, approximately 12 hours later, the embryo cleaves to the 8-cell stage (Fig. 1) (Hogan *et al.,* 1994).

Unlike somatic cells, the cell cycles of the early cleavage-stage embryo do not feature growth phases in which cell size increases. Instead, the early embryo undergoes "reductive cleavages" in which the total volume of the embryo remains unchanged, so that each cell is one-half the volume of the cell which gave rise to it. In this way, progressively smaller cells termed blastomeres are produced by each successive cleavage through at least the 16-cell stage (Lehtonen, 1980).

The blastomeres of the cleavage-stage embryo are in contact with one another and are contained within a spherical shell of extracellular matrix called the zona pellucida (Dean, 1983). However, blastomeres are not connected by cell adhesion molecules or gap junctions, and thus do not directly communicate or form an integrated tissue.

Compaction

The mid-eight cell embryo, which in the mouse occurs on day three post-fertilization, marks the end of the cleavage stages (Fig.1). It is during this stage that the morphogenetic process of compaction occurs. During compaction the embryo progresses from a collection of rounded, loosely associated blastomeres into a roughly spherical embryo in which distinct cell boundaries are not clearly visible (Sutherland et. al., 1983). It is also the first event leading to cellular differentiation in the embryo (Johnson et al., 1986). During compaction, the plasma membrane of adjacent cells come in such close apposition that their boundaries become obscured when observed under light microscopy (Ducibella and Anderson, 1975), which makes detection of this event easy at the light microscopic level.

The first step of compaction involves an increased Ca^{++}-dependent adhesion between blastomeres (Ducibella and Anderson, 1975) mediated by E-cadherin (Kidder, 1992; Fleming et al., 2001). E-cadherin is a transmembrane domain glycoprotein with two highly conserved Ca^{++} binding motifs (Takeichi, 1991; Kelmer 1993). During cell to cell adhesion, E-cadherin is present along all regions of cell contact (Fleming et. al. 2001). E-cadherin is concentrated at the adherens junction and interacts homophilically with E-cadherin molecules of adjacent cells to engage in extracellular adhesion (Aberle et. al., 1996; Fleming et al., 2001). E-cadherin is also involved in intracellular adhesion to the actin cytoskeleton through its ability to form complexes with intracellular catenin proteins (Kemler, 1993; Fleming et. al. 2001). Catenin proteins act as actin-binding proteins and mediate the connection to actin filaments (Kemler, 1993).

By day 3.5 post-fertilization in mouse, the compacted blastomeres form a round sphere of compacted cells called a morula (Brinster, 1974). At this stage individual blastomeres can no longer be observed and two types of intracellular junctions are formed.

The first are gap junctions that develop between individual blastomeres allowing for intracellular communication between all cells within the embryo (Ducibella, 1975; Lo, 1979). Gap junctions are specialized organelles consisting of clustered channels called connexons (Bruzzone et. al., 1996, Houghton 2005). A connexon from one cell docks in the intracellular space with the connexon from the adjoining cell to form a complete gap junction channel (Houghton, 2005). Each connexon is made up of six connexin proteins. Preimplantation mouse embryos form homomeric gap junctions comprised of connexin 43 (Kidder, 1992; Houghton, 2005). Gap junctions allow for the exchange of molecules up to about 1 kD in size between adjacent cells without secretion or leakage into the extracellular space (Bruzzone et. al., 1996) allowing for cellular homeostasis and intercellular communication.

The second type of junctions formed are zonular tight junctions, which form between the apical surfaces of the outermost cells to create an outer epithelial layer that restricts permeability from the external environment into the embryo (Ducibella, 1975; Lo 1979). This marks the start of the functional differentiation of the two different cell lineages that arise in the preimplantation embryo. The outer layer forms the epithelial trophectoderm, which gives rise to the trophoblast lineage. The inner cells will form the epiblast lineage that gives rise to the fetus.

Between 3.5 and 4.5 days post-fertilization, the inner and outer cells take on distinct characteristics, and the embryo forms three different compartments. The outer trophectoderm is a fluid-transporting epithelium, which transports water and solutes into the spaces between the inner cells creating the fluid-filled blastocoel cavity (Hardy et al., 1989). The inner cells, now termed the inner cell mass, remain as a group of cells adhering to the inner surface of the trophectoderm, within the blastocoel cavity. The cells of the inner cell mass are embryonic stem cells, and will give rise to the fetus (Watson, 1992). The trophectoderm gives rise to trophoblast that develops into fetal membranes and the fetal portion of the placenta. Fluid transport by the trophectoderm continues until the blastocoel is fully expanded and the trophectoderm forms a stretched spherical shell around the blastocoel (Dean, 1983).

When the blastocoel forms, the embryo becomes a blastocyst (Fig 1). Blastocysts begin with a small cavity that is not much expanded. However, as fluid accumulation continues, the blastocyst becomes fully expanded and stretches the surrounding zona pellucida, so that it becomes noticeably thinned. The fully-expanded blastocyst finally hatches from the zona pellucida, and the trophectoderm comes into contact with the uterine endometrium (Tortara and Grabowski, 2000).

Preimplantation Embryo Pathway

Reproduction first begins in the ovary where follicles grow and eventually release oocytes. Follicular growth and ovulation are controlled by gonadotropin releasing hormone (GnRH) excreted from the hypothalamus. GnRH promotes the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary.

FSH promotes follicular growth, producing a mature or Graafian follicle. LH aids in the development of the mature follicle and causes the rupture of the Graafian follicle and the release of the oocyte (Raga et al., 1999). Once the egg is released from the follicle it is deposited into a muscular structure called the Fallopian tube or oviduct. The ampulla, the widest portion of the oviduct, is the location where fertilization occurs (Crow et al., 1994). The cleavage stages of the embryo (1 cell to 8 cell) develop within the oviduct as the embryo moves toward the uterus. At the utero-tubal junction, between the oviduct and the uterus, compaction occurs and a morula is created. Past the utero-tubal junction, the blastocyst forms within the uterus where implantation occurs (Dard et al., 2008).

Implantation

Once the blastocyst hatches from the zona it is able to implant into the uterine wall. Escape from the zona pellucida is necessary for the process of implantation since the blastocyst's trophoblast cells create the embryonic tissue responsible for establishing embryonic contact with the mother (Paria et. al., 2001). The first event of implantation is apposition. During apposition the trophectoderm of the blastocyst becomes closely apposed to the luminal epithelium of the uterus (Wang and Dey, 2006). At the site of apposition there is an increase in uterine stromal vascular permeability (Carson et. al., 2000). Ovarian steroids E2 and/or P4 mediate this change on the surface of the uterine epithelium (Lee and DeMayo, 2004). During preimplantation embryo development surges in P4 levels initiate uterine stromal cell proliferation while secretion of low doses of E2 further stimulates uterine stromal cell proliferation and differentiation (Lee and DeMayo, 2004). These changes in the uterine epithelium make it a receptive surface for

blastocyst attachment, the next step of implantation. Attachment of the blastocyst to the uterus stimulates decidualization, a process in which uterine stromal cells proliferate and differentiate into a spongy mass of cells termed decidual cells (Carson et. al., 2000; Dey et. al. 2006). Once the blastocyst attaches to the uterus, its epithelial cells undergo apoptosis allowing the trophoblasts to penetrate the decidual tissue (Carson et. al. 2000).

After implantation, cells within the embryo continue to proliferate and differentiate. The inner cell mass (epiblast) develops into the embryo proper while the cells of the inner cell mass in contact with the blastocoel develop into the primitive endoderm (Adamson and Gardner, 1979). The polar trophoblasts differentiate into the chorion, which facilitates further penetration of the blastocyst into the uterine lining and the transfer of nutrients from maternal blood to fetal blood via chorionic villi. A portion of the chorion will continue to penetrate into the endometrium forming the placenta (Cross, 2005). While implanted in the uterus, fetal development is completed within a species-specific gestation period.

In Vitro Development of Preimplantation Mouse Embryos

Preimplantation mouse embryo development can occur in vitro in chemically defined media. This was first demonstrated by Whitten in 1956 when he reported that 8-cell mouse embryos could develop into blastocysts when cultured in a simple chemical medium containing only glucose, bovine serum albumin, bicarbonate and salts. In 1958, McLaren and Biggers reported that mouse blastocysts, developed in Whitten's medium from the 8-cell stage, could develop into live young once transferred to a female recipient. Over approximately the next decade, improvements to culture medium,

primarily consisting of the addition of lactate and pyruvate, permitted in vitro development of mouse embryos from each stage (Biggers, 1998).

However, in vitro a phenomenon called the "two-cell block" is observed in which one-cell zygotes from females of most genotypes undergo a single division and are arrested at the two-cell stage, even though 2-cell embryos are able to develop to blastocysts in the same medium (Biggers, 1998). The cause of the "two-cell block" is unknown. The block could be caused by intrinsic factors arising from the embryo or by environmental factors present in the culture media. It was demonstrated that the two-cell block could be alleviated by transferring embryos to organ cultures of the oviduct (Biggers et al., 1962) or to the oviduct of pseudopregnant females (Whittingham and Biggers, 1967). Therefore it was hypothesized that the oviduct provided a unique microenvironment for the preimplantation embryo (Biggers, 1998). Yet it was observed that the occurrence of the "two-cell block" is strain dependant, since zygotes from outbred and most inbred females expressed the "two-cell block" while zygotes of F_1 hybrids between inbred strains did not (Whitten and Biggers, 1968).

However, in the late 1980s an organized effort to optimize culture media resulted in alleviating the "two-cell block" in blocking-type mouse embryos. Alterations to the chemically defined media used by Whitten (1956), including the addition of ethylenediaminetetraacetic acid (EDTA) (Abramczuk et al., 1977), the addition of glutamine (Chatot et al., 1989), a lower concentration of phosphate and glucose, an increase in the concentration of K^+, and a decrease in NaCl, led to the alleviation of the "two-cell block" (Erbach et al., 1995). As a result of these findings several different media have been developed to support the culture of mouse embryos susceptible to the

"two-cell block", including CZB and KSOM (Biggers, 1998). Such media capable of alleviating the "two-cell block" had lower concentrations of many components including glucose and Na^+ than previous media (Biggers 1998). These alterations to the media resulted in a much lower osmolarity, raising the possibility that the "two-cell block" could be induced at least in part by high osmolarity. This idea was supported when Hadi et al. (2005) demonstrated that both blocking strain and non-blocking strain mice became blocked at the 2-cell stage when osmolarity was increased, although higher osmolarity was required in the non-blocking strain. Therefore media capable of overcoming the "two-cell block" generally have lower osmolarities than media in which the "two-cell block" occurs (Baltz, 2001).

Osmolarity and In Vitro Mouse Embryo Culture

Cleavage-stage mouse preimplantation embryos are highly sensitive to increased external osmolarity. Osmolarity is the concentration of osmotically active particles expressed in terms of osmoles of solute per liter of solution (mOsM). When cultured from zygotes, most preimplantation mouse embryos will not develop in vitro in media with an osmolarity above 300 mOsM and exhibit the "two-cell block". Culture media such as CZB at 275 mOsM and KSOM at 250 mOsM are successful at overcoming the "two-cell block" (Devreker and Hardy, 1997). Cultured in media with an osmolarity of 310 mOsM, fewer than about 10% of mouse zygotes from CF1 females, which are susceptible to the 2-cell block, will develop to the blastocyst stage (Dawson and Baltz, 1997). However the osmolarity of mouse oviductal fluid is approximately 290-300

mOsM (Collins and Baltz, 1999), similar to blood. Therefore, mouse oviductal fluid must have properties that enable the embryos to survive at these higher osmolarities.

Organic Osmolytes in Embryo Culture

One mechanism by which embryos could develop at high osmolarities within the oviduct might be through the use of organic osmolytes. As described in the section below on cell volume regulation, a number of cell types have been found to accumulate any of a number of small, uncharged organic compounds to displace intracellular ions and thus provide intracellular osmotic support while avoiding the damaging effects of high intracellular ionic strength.

It has been demonstrated that early preimplantation mouse embryos would develop from zygotes through the 2-cell block at higher osmolarities in vitro when any of several potential organic osmolytes were added to the culture media (Dawson and Baltz, 1997; Gardner and Lane, 1999, Van Winkle, 2001). Organic osmolytes shown to protect the development of cleavage-stage mouse embryos in culture against increased osmolarity include proline (Dawson and Baltz, 1997), alanine (Van Winkle et al., 1990a), glutamine (Lawitts and Biggers, 1992; Dawson and Baltz, 1997), glycine (Van Winkle et al., 1990a; Dawson and Baltz, 1997), β-alanine (Dawson and Baltz, 1997; Hammer and Baltz, 2003) and betaine (Biggers et. al. 1993; Hammer and Baltz, 2002),

Proline (tested at 1 mM) is highly effective at providing osmoprotection to embryo development from the 1-cell to blastocyst stage (Dawson and Baltz, 1997). Proline is a good candidate for an endogenous organic osmolyte used by preimplantation embryos since it is found in mouse oviductal fluid (Aguiler and Reyley, 2005).

Alanine is able to support 2-cell embryo development to the blastocyst stage in media with increased osmolarity (Van Winkle et al., 1990a), but whether it functions from the zygote stage has not been assessed.

Lawitts and Biggers (1992) first proposed that glutamine protects mouse cleavage-stage embryos against high osmolarities by acting as an organic osmolyte. It was observed that glutamine alleviated the detrimental effects of increased NaCl concentration on mouse zygote development (Lawitts and Biggers, 1992). In addition Dawson and Baltz (1997) demonstrated that glutamine at a concentration of 1 mM protected cleavage-stage embryos development against high osmolarity whether osmolarity was raised using NaCl or by added raffinose. This indicates that the osmoprotective effect of glutamine is independent of substantial NaCl concentration (Dawson and Baltz, 1997). Therefore glutamine protects against increased osmolarity rather than NaCl concentration.

Glycine also protects cleavage-stage mouse embryos against hypertonicity. Van Winkle et al. (1990a) observed that glycine alleviated the detrimental effects of high osmolarity on the development of two-cell embryos to blastocysts. Also, Dawson and Baltz (1997) concluded that glycine has a significant protective effect on the development of zygotes to the blastocyst stage in media whose osmolarity had been increased by NaCl or raffinose. Maximal protection of mouse cleavage-stage embryo development was reached at very low glycine concentrations with a half-maximally effective concentration of 50 μM (Dawson and Baltz, 1997; Baltz, 2001). Dawson et al. (1998) demonstrated that one-cell mouse embryos could maintain their volumes against increased osmolarity in the presence of glycine. Steeves et al. (2003) determined that the intracellular accumulation

of glycine in cleavage-stage embryos is regulated by osmolarity. Glycine is present in mouse eggs, cleavage-stage embryos and oviductal fluid (Shultz et al., 1981, Aguiler and Reyley, 2005) making it a good candidate as an endogenous organic osmolyte (Baltz, 2001).

β-Alanine is effective at protecting one-cell mouse embryo development to blastocysts at increased osmolarity (Dawson and Baltz, 1997). β-Alanine confers maximal protective effect on cleavage-stage embryo development, however, only at a high concentration of 5 mM (Dawson and Baltz, 1997; Baltz, 2001). Hammer and Baltz (2003) found that β-alanine could function as an organic osmolyte in mouse embryos, since it is osmoprotective and its accumulation is increased by increased osmolarity.

Betaine is also highly effective at protecting cleavage-stage embryo development against increased osmolarity (Biggers et al. 1993; Hammer and Baltz, 2002). Betaine is effective at low concentrations with half-maximal protection of one-cell embryo development to blastocysts at about 40 μM (Hammer and Baltz, 2002). Like glycine and proline, betaine is a good candidate for an endogenous organic osmolyte use by preimplantation embryos since it is found in mouse oviductal fluid and its concentration in oviductal fluid is higher post-fertilization (Anas et al. 2007).

Not all of the potential organic osmolytes found to be effective in vitro may be also functioning in vivo. For example, glycine is able to inhibit β-alanine accumulation in cleavage-stage embryos (Hammer and Baltz, 2003), while neither betaine nor β-alanine are able to inhibit glycine accumulation in preimplantation embryos (Dawson et al., 1998). Therefore, early cleavage-stage mouse embryos may preferentially accumulate glycine as the major physiological organic osmolyte. However, the relative contributions

of each potential organic osmolyte in zygotes and early cleavage-stage embryos has not been systematically assessed.

Table I: Potential organic osmolytes that will be tested in this study.

Organic Osmolyte	Protective Capabilities	Transporter in Cleavage-Stage Embryos	Transporter in Somatic Cells
Glutamine	Protects one-cell embryo development from ↑ osmolarity	GLYT1	System A transporter
Glycine	Protects one-cell embryo development from ↑ osmolarity	GLYT1	System A transporter
Betaine	Protects one-cell embryo development from ↑ osmolarity	Betaine/proline transport system	Betaine transporter (BGT1) or System A transporter
Proline	Protects one-cell embryo development from ↑ osmolarity	Betaine/proline transport system	System A transporter
β-Alanine	Protects one-cell embryo development from ↑ osmolarity	β transporter	β transporter
Taurine	Protects one-cell embryo development to the 4-cell stage from ↑ osmolarity	β transporter	β transporter
Myo-Inositol	Does not protect one-cell embryo development from ↑ osmolarity	N/A	Myo-inositol transporter
L-Alanine	not determined	very little transport in early embryos	System A transporter

Cell Volume Regulation

Like mouse preimplantation embryos, many mammalian somatic cells are sensitive to increased osmolarity (Hallows and Knauf, 1994). Mammalian somatic cells act as nearly ideal osmometers. Therefore, when placed in a hypertonic environment, animal cells cannot withstand the osmotic pressure differential between the intracellular and extracellular environments (Baltz, 2001). Thus, mammalian somatic cells passively lose water to the outside hypertonic environment until the osmotic pressure differential is eliminated, resulting in a decrease in cell volume (Lang *et al.,* 1998).

In order to regain their normal volume, mammalian cells undergo a process known as regulatory volume increase (RVI) (Fig. 1). During short-term RVI the cell immediately accumulates inorganic ions to exert osmotic pressure within the cell and return the cell to its normal volume (Hallows and Knauf, 1994, Lang *et al.*, 1998). This is accomplished by activating inorganic ion transporters such as Na^+/H^+ exchangers, HCO_3^-/Cl^- exchangers, and K^+/Cl^- cotransporters that remain activated until the cell regains the normal volume.

In some cells, the inorganic ions are then more slowly replaced by an array of organic compounds, collectively termed "organic osmolytes," as a source of osmotic support (Lang *et al.*, 1998). The accumulation of organic osmolytes is a slow process occurring after several hours or days of continued exposure to hypertonicity (Kwon and Handler, 1995). During this lag time the hypertonic environment initiates the increased transcription and subsequent translation of the organic osmolyte transporters (Kwon and Handler, 1995).

The replacement of inorganic ions by organic osmolytes is crucial for cell viability under conditions where an excessive level of intracellular ions would be required to balance external osmolarity or to support normal cell volume. Inorganic ions can have destabilizing effects on macromolecules within the cell. The replacement of inorganic ions with organic osmolytes counteracts this negative effect (Wehner et al., 2003). Unlike inorganic ions, organic osmolytes, even at extremely high concentrations are compatible with normal macromolecular function, since they do not interfere with cellular biochemistry nor destabilize macromolecules (Yancey, 2005).

Intracellular accumulation of organic osmolytes most often involves uptake across the cell membrane by specific transport systems (Wehner et al., 2003). Four organic osmolyte transporters have been identified in mammalian cells: myo-inositol transporter, system β transporter, system A transporter and betaine/GABA transporter.

The betaine transporter is part of the neurotransmitter transporter gene family (Schloss, 1994). Therefore the accumulation of betaine as an organic osmolyte via the betaine transport system is coupled with Na^+ and Cl^- cotransport (Kwon, 1995). The betaine/GABA transporter protein BGT1 encoded by the *SLC6A12* gene is in the GABA transporter subfamily (Chen et al., 2004). It is the only GABA transporter isoform that also transports betaine (Kwon, 1995).

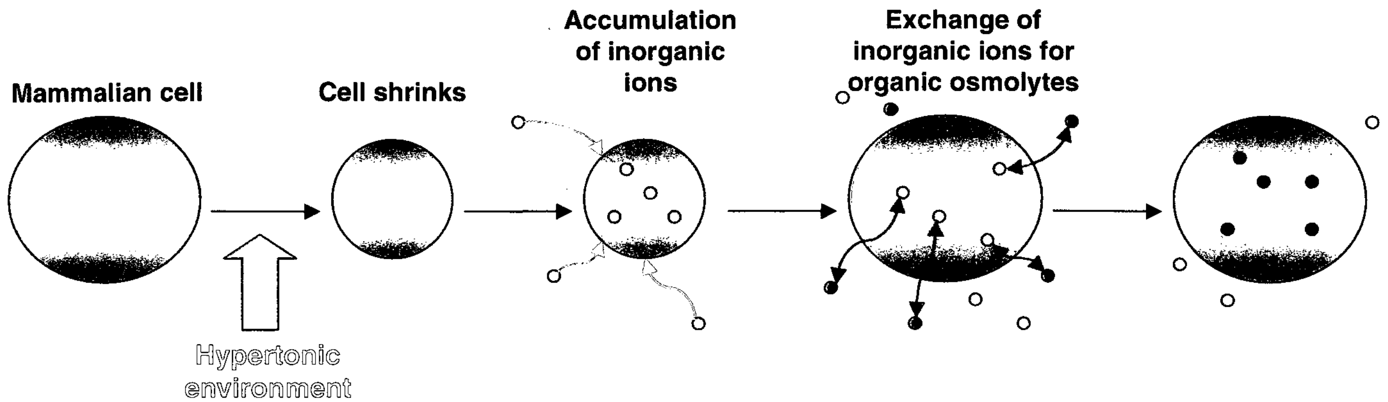
The myo-inositol transporter is part of the Na^+ coupled glucose transporter family (Kwon et al., 1992). The myo-inositol transporter protein SMIT is encoded by the *SLC5A3* gene and has the ability to concentrate myo-inositol in cells to be used as an organic osmolyte (Kwon 1995; Kwon et al. 1992; Wright and Turk, 2004).

System A is responsible for the accumulation of neutral amino acids as organic osmolytes (Chen and Kempson, 1995). The system A transporter protein ATA2 is encoded by the *SLC38A2* gene and performs Na^+ coupled transport of neutral amino acids (Pastor-Anglada et al., 1996; Mackenzie and Erickson, 2004) such as alanine, methionine, proline, and serine.

The taurine or system β transporter is widely expressed in many tissues and is also a member of the sodium and chloride-coupled neurotransmitter transporter gene family (Kwon and Handler, 1995). The taurine transporter protein NCT/TAUT is encoded by the *SLC6A6* gene and transports taurine along with many other β-amino acids including β-alanine (Kwon and Handler, 1995; Chen et al., 2004). In many cells, taurine functions as an organic osmolyte, and β-alanine serves this function in a number of cells as well.

Figure 2: Regulatory Volume Increase

Regulatory volume increase is the ability of cells to regain normal volume after a volume loss. In the short term cells accumulate inorganic ions in order to increase osmotic pressure and therefore increase cell volume. Cells slowly exchange inorganic ions for organic osmolytes as a source of osmotic support and to maintain long term regulatory volume increase.



Cell Volume Regulation in Cleavage-Stage Embryos

Like mammalian somatic cells, early preimplantation mouse embryos utilize transporters to accumulate organic osmolytes. However preimplantation mouse embryos do not use the four known organic osmolyte transport systems found in mammalian somatic cells. Of the four known transport systems, only the system β transporter is present throughout preimplantation development (Van Winkle, 1996). However system β is not important for cell volume regulation or osmoprotection (Hammer and Baltz, 2003) and instead performs other physiological functions for the embryo.

Instead, mouse preimplantation embryos utilize a transport system, glycine transporter 1 (GLYT1; *SLC6A9* gene) as a novel organic osmolyte transporter (Steeves et al. 2003). GLYT1 is a sodium and chloride dependent member of the neurotransmitter transporter family (Schloss, 1994). GLYT1 was originally shown to be active in 1-cell and 2-cell mouse embryos and has a high affinity for its substrate glycine (Steeves, 2003). As previously mentioned, glycine acts as an organic osmolyte in preimplantation mouse embryos and is preferentially accumulated in mouse cleavage-stage embryos. In the absence of glycine one-cell mouse embryos will not develop in the presence of high osmolarity (Dawson, 1997) and cannot maintain their volumes (Steeves et al., 2003). Using the GLYT1 specific inhibitor ORG23798, Steeves et al. (2003) demonstrated that when GLYT1 activity was inhibited, 1-cell mouse embryos could not maintain their volume at high osmolarities even in the presence glycine. Steeves et al. (2003) also discovered that one-cell mouse embryos could not develop past the 2-cell stage in the face of increased osmolarity when GLYT1 was inhibited by ORG23798, even in the presence of glycine. Therefore GLYT1 was shown to play an essential role in cell volume

regulation and osmoprotection in 1-cell mouse embryos, which are distinct characteristics of an organic osmolyte transporter. Unlike the four mammalian organic osmolyte transporters previously described, hypertonic stimulation of glycine accumulation via GLYT1 activity has no apparent lag time and is not dependant on transcription and translation (Steeves et al., 2003).

Mouse preimplantation embryos may also possess a similar sodium and chloride-dependant organic osmolyte transporter shared by betaine and proline (Anas et. al. 2007). Both betaine and proline are actively transported into cleavage-stage embryos where they act as organic osmolytes, protecting embryo development from the 1-cell stage at increased osmolarity (Biggers et al., 1993, Dawson and Baltz, 1997). Using inhibition profiles and kinetics measurements, Anas et al. (2007) demonstrated that betaine and proline are transported by a single shared transport mechanism in 1-cell and 2-cell mouse embryos. An increase in external osmolarity caused a moderate increase in betaine and proline accumulation in one-cell mouse embryos over a 24 hour period (Anas et al., 2007). Also similar to GLYT1, the betaine/proline transporter activity has no apparent lag time and is immediately up-regulated in the presence of increased osmolarity (Anas et al., 2007). Subsequently, this transport system was identified as the betaine/proline transporter protein SIT1, encoded by the *SLC6A20* gene, and was found to be active only at the 1-cell and 2-cell stages (Anas et al, 2008).

Osmoprotection by the GLYT1 transport system and the SIT1 betaine/proline transport system in mouse preimplantation embryos disappears by the four cell stage (Anas et al. 2008). Hammer et al. (2000) reported that mouse embryos cultured from the two-cell to the eight-cell stage in the presence of glycine showed a significantly increased

amount of glycine accumulation at high osmolarity. However, there was no effect of increased osmolarity on glycine accumulation by mouse embryos cultured from the eight-cell stage to the blastocyst stage. Therefore, osmoregulated GLYT1 activity appears to be present only in cleavage-stage mouse embryos and is no longer active by the 8-cell stage (Hammer et al., 2000, Van Winkle et al., 1988). Anas et al. (2008) demonstrated that the novel betaine/proline transporter is active in the one-cell and two-cell mouse embryos in the presence of high osmolarity, however it is no longer active by the 4-cell stage. Therefore, during the mouse embryo preimplantation period, both GLYT1 and the betaine/proline transporter activity are present only during the cleavage-stages.

It is not known whether preimplantation mouse embryos are sensitive to increased osmolarity when GLYT1 is no longer active. Whether increased osmolarity is detrimental to post-compaction stage embryo development therefore still needs to be studied. Although protection of embryo development from high osmolarity by various organic osmolytes has been demonstrated, all previously- reported work involved pre-compaction, cleavage stage embryos and there is therefore still a question as to whether organic osmolytes are used by post-compaction stage embryos.

Rationale for Study

It is not known how sensitive embryos are to increased osmolarity in the peri-compaction and post-compaction periods. Testing whether post-compaction stage mouse embryos are sensitive to increased osmolarity, and determining the threshold for detrimental effects on post-compaction embryo development, would help to determine if they likely require osmoregulatory mechanisms similar to those of cleavage-stage

embryos. Determining whether embryo development is negatively affected by osmolarities close to those they experience in vivo will reveal whether they likely need specialized mechanisms for alleviating osmotic stress, such as organic osmolyte transport and accumulation. Investigating potential organic osmolytes in post-compaction stage embryos will aid in determining what mechanisms are used to alleviate any detrimental effects of increased osmolarity. Therefore a better understanding of post-compaction stage embryo physiology will be provided. In addition, this would help define better conditions for producing healthy embryos in vitro. Even if organic osmolytes are not found to be important at normal osmolarities, they could nonetheless become important in vitro under conditions of osmotic stress such as encountered during cryopreservation.

OBJECTIVES

The overall objective of the study is to determine the threshold above which increased osmolarity is detrimental to post-compaction embryo development, and the osmoprotective abilities of potential organic osmolytes on post-compaction-stage embryos.

Objectives and Hypotheses

Objectives:

1. To determine the activity of the GLYT1 organic osmolyte transporter at various stages of mouse preimplantation embryo development and show that it is no longer active in post-compaction embryos.

2. To determine the range of osmolarities that are permissive for embryo development from the 8-cell stage to blastocyst, in the absence of possible organic osmolytes.
3. To identify potential organic osmolytes that are effective in alleviating the detrimental effects of increased osmolarity on compaction-stage embryos.
4. To identify possible transport mechanisms used by any organic osmolytes found to be effective in protecting compaction-stage embryos.

Hypotheses:

1. The GLYT1 organic osmolyte transporter will be active in embryos before compaction but not after.
2. Post-compaction embryos will be sensitive to increased osmolarity and fails to develop normally when osmolarity is increased above a certain point.
3. A subset of potential organic osmolytes will protect compaction-stage embryos against increased osmolarity when added to the culture medium.
4. Effective organic osmolytes will be accumulated in compaction-stage embryos via specific transporters.

MATERIALS AND METHODS

Chemicals and Media

Chemicals

Media components and mineral oil used in culture were embryo-tested or cell culture grade. Components of media for culturing embryos, including organic osmolytes, and mineral oil were purchased from Sigma (St. Louis, MO, USA). Hyaluronidase was purchased from Sigma (St. Louis, MO, USA). Scintiverse BD scintillation fluid was purchased from Fisher Scientific (Pittsburgh, PA, USA). Acid Tyrode solution was mouse embryos tested and purchased from Sigma (St. Louis, MO, USA). The DNA-binding dye Bisbenzimide (Hoechst 33258) was obtained from Sigma (St. Louis, MO, USA).

GLYT1 specific inhibitor

The GLYT1 transport inhibitor ORG23798 [bis(4-fluorophenyl) methylenepiperidineacetic acid, lithium salt] was synthesized at Organon Laboratories, UK, and was a gift of Dr. Richard Armer. Its validation and use with embryos has been extensively described previously (Steeves et al., 2003). A 1000x stock was prepared in DMSO and stored at -20°C until used.

Tritiated compounds

^{3}H-labelled amino acids were obtained from Amersham Biosciences (Arlington Heights, IL, USA) and include: ^{3}H-glycine ([2-^{3}H]glycine; 14–20 Ci/mmol), ^{3}H-

glutamine (l-[G-^{3}H]glutamine; 59 Ci/mmol), ^{3}H-alanine (L-[2,3-^{3}H]alanine; 47 Ci/mmol), and ^{3}H-taurine ([1,2-^{3}H]Taurine; 27 Ci/mmol).

Solutions

Culture media

Embryo culture media used were based on KSOM medium (Lawitts and Biggers, 1993), with glutamine omitted. Polyvinyl alcohol (PVA; 1 mg/ml) was substituted for BSA as the macromolecular component of the medium since BSA has the potential to contribute amino acids to the medium and carries other unknown contaminants that could affect results (Dawson and Baltz, 1997). The components of the KSOM medium used were (in mM) NaCl (95), KCl (2.5), KH_2PO_4 (0.35), $MgSO_4 \cdot 7H_2O$ (0.2), Na lactate (10), glucose (0.2), Na pyruvate (0.2), $NaHCO_3$ (25), CaCl2 (1.7), EDTA (0.1), K penicillin G (0.16) and streptomycin (0.03). The osmolarity of KSOM, 250 mOsM, was verified within ± 5 mOsM using a vapor pressure osmometer (Vapro 5520, Wescor, Logan, UT).

Media for osmolarity measurements

Media were based on KSOM embryo culture medium as described above. The osmolarity of the culture media was varied up to 450 mOsM by adding the appropriate concentrations of the trisaccharide D(+)-raffinose to the base culture media. Raffinose is used because it is not metabolized or transported by mammalian cells and therefore functions as an inert external osmolyte in this system. Osmolarity was confirmed to be within ± 5 mOsM of the nominal value using the vapor pressure osmometer (Dawson and Baltz, 1997).

Embryo handling medium

Hepes-KSOM was used as the collection media, in which all but 4 mM of the $NaHCO_3$ in KSOM was replaced with Hepes and the pH adjusted to approximately 7.3 using NaOH (Lawitts and Biggers). The osmolarity of Hepes-KSOM, 240 mOsM, was verified within ± 5 mOsM using the vapor pressure osmometer.

Staining medium

Staining medium used was based on Hepes-KSOM in which 50 μg of Bisbenzimide (Hoechst 33258) was added per 1 mL of Hepes-KSOM (Ebert et al., 1985). Bisbenzamide was added from a 1000x stock prepared in embryo-grade water and stored at 4°C (up to 1 month) until used.

Organic osmolyte stocks

Potential organic osmolytes used in culture were embryo-tested or cell culture-tested and were purchased from Sigma (St. Louis, MO). Where specified, the potential organic osmolytes (glutamine, glycine, betaine, proline, taurine, β-alanine, myo-inositol, and L-alanine) were added to the medium. Potential organic osmolytes were added directly to modified KSOM from 100 mM stocks that were freshly thawed after being stored at -20°C for no more than one month, as previously described (Dawson and Baltz, 1997) to either 1 mM or 5 mM final concentration, as specified.

Embryos

Embryos were obtained from female CF1 mice (Charles River, St-Constant, PQ, Canada) between 4-6 weeks of age. Embryos were isolated from female mice that had been superovulated with an intra-peritoneal injection of 5 IU of pregnant mare's serum gonadotropin (PMSG) followed 48 hours later by 5IU of human chorionic gonadotropin (hCG) and then caged with BDF males overnight (Dawson and Baltz, 1997). Embryos were extracted by flushing the excised oviduct and/or uterus with collection media after the females had been killed by cervical dislocation. 1-cell embryos were extracted approximately 15 hours post-hCG and separated from the cumulus cells with hyaluronidase. 2-cell embryos were collected approximately 2 days post-hCG, 4-cell embryos 2.5 days post-hCG, 8-cell embryos 3 days post-hCG, morula 3.5 days post-hCG and blastocysts 4 days post-hCG. All animal protocols were approved by the Animal Care Committee of the Ottawa Hospital Research Institute.

Embryo Culture

Embryos were collected in Petri dishes, washed, and placed into microdrops of culture media that had been pre-incubated under mineral oil at 37°C and 5% CO2 in tissue culture dishes, using flame-pulled, mouth-operated pipettes. The day embryos are placed into culture is considered day 1. BD Falcon organ, Petri and tissue culture dishes were all obtained from VWR International (Arlington Heights, IL, USA). The specific protocols used for embryo culture are given below.

Measuring development of 8-cell embryos to blastocysts as a function of osmolarity

Embryos at the 8-cell stage were isolated from CF1 females 3 days post-hCG. Embryos were placed in microdrops of culture media of different osmolarities (250 mOsM, 300 mOsM, 310 mOsM, 360 mOsM, 370 mOsM, 390 mOsM, 400 mOsM, 410 mOsM, and 450 mOsM) that had been pre-incubated under oil at 37°C and 5% CO_2 for 24 hours. 8-cell embryos were placed into culture (day 1) and the proportion reaching the blastocyst stage on day 3 and day 4 of culture was recorded. Embryos were photographed using a digital camera on a Zeiss Axiovert inverted microscope using the 20X objective on day 2, 3 and 4 of culture. A picture of a 2 mm scale was taken prior to the embryo pictures with the same camera and microscope settings for calibration.

Measuring the effectiveness of potential organic osmolytes in promoting mouse blastocyst development from the 8-stage

Embryos at the 8-cell stage were isolated from CF1 females 3 days post-hCG. Embryos were placed in microdrops of modified KSOM at 400 mOsM with the addition of 1 mM of organic osmolyte (glutamine, glycine, betaine, proline, taurine, myo-inositol, L-alanine or β-alanine) or 5 mM of organic osmolyte (β-alanine) and KSOM at 250 mOsM and 400 mOsM without organic osmolyte as a control. Microdrops had been pre-incubated under oil at 37°C and 5% CO_2 for 24 hours. 8-cell embryos were placed into culture (day 1) and the proportion reaching the blastocyst stage on day 3 and day 4 of culture was recorded.

Measuring the effect of potential organic osmolytes on the size of mouse blastocyst developed from the 8-stage as a function of osmolarity

Embryos at the 8-cell stage were isolated from CF1 females 3 days post-hCG. Culture media used were based on KSOM at varying osmolarities (250 mOsM, 310 mOsM, 340 mOsM, 370 mOsM and 400 mOsM), and contained either no organic osmolyte or 1 mM of organic osmolyte (glutamine, glycine, L-alanine) or 5 mM of organic osmolyte (β-alanine). Embryos were placed in microdrops of culture media that had been pre-incubated under oil at 37°C and 5% CO_2 for 24 hours. 8-cell embryos were placed into culture (day 1) and the proportion reaching the blastocyst stage on day 3 and day 4 of culture was recorded. Embryos were photographed using a digital camera on an inverted microscope a Zeiss Axiovert microscope using the 20X objective on day 2, 3 and 4 of culture. A picture of a 2 mm scale was taken prior to the embryo pictures with the same camera and microscope settings. From these digital photos the size of each blastocyst (see below) as a function of osmolarity was ascertained.

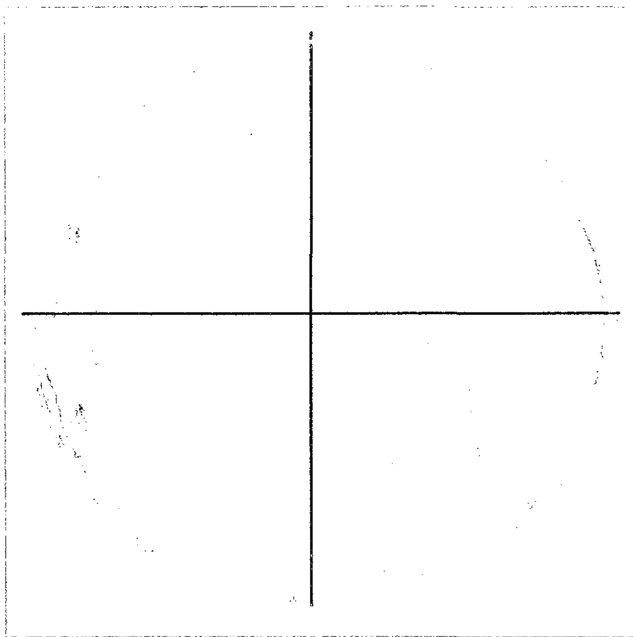
Measuring the Dimensions and Calculating Volumes of Blastocyst

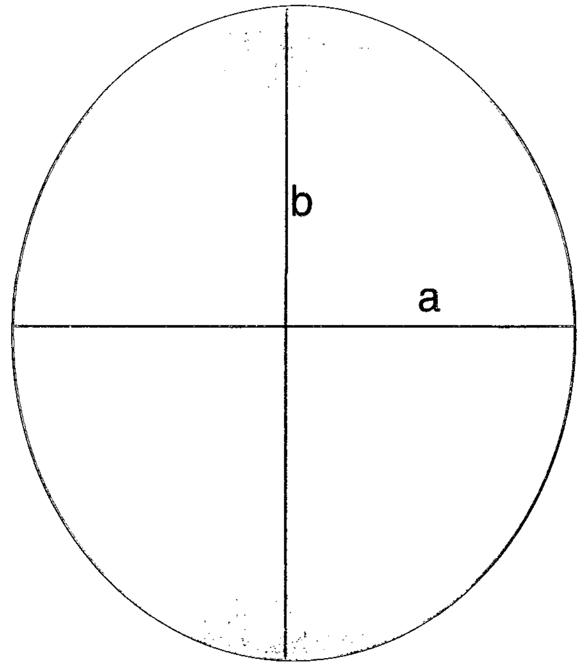
As stated above digital photos were taken of blastocysts developed from the 8-cell stage at varying osmolarities (250 mOsM, 310 mOsM, 340 mOsM, 370 mOsM and 400 mOsM) in the presence or absence of potential organic osmolyte (1 mM of glutamine, glycine or L-alanine or 5 mM of β-alanine). The dimensions of mouse embryos in digital photos were measured using ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Embryo volumes were determined by measuring two orthogonal radii and using

them to calculate the volume of the embryo, assumed to be an ellipsoid (Fig. 2) of volume [$V = (4/3)\pi(a)^2(b)$].

Figure 3: Embryo Volume Calculation

In order to calculate mouse embryo volume the shape of the blastocyst was assumed to be a prolate ellipsoid. The two orthogonal radii were measured (major radius b and minor radius a) and the volume calculated using the following equation: $V = (4/3)\pi(a)^2(b)$





^{3}H-Labeled Compound Measurements in Embryos

^{3}H-labeled amino acids were added directly to media at a concentration of 1 μM. Groups of 5 to 10 embryos were rinsed three times in KSOM that had been pre-incubated under oil at 37°C and 5% CO_2 for 24 hours. The embryos were transferred to KSOM containing ^{3}H-labeled organic osmolyte and incubated at 37°C and 5% CO_2 under oil in culture dishes for 10 minutes unless otherwise specified. Following incubation, the embryos were washed through several drops of ice-cold collection media and transferred to a scintillation vial. Collection media from the last wash drop was transferred to a separate scintillation vial in order to measure the background radioactivity. 4 mL of scintillation fluid is added to each vial and the total tritium label in the embryos was determined by scintillation counting (2200CA TriCarb, Packard Instrument). Using a standard curve, the counts per minute were converted to the number of moles of radiolabeled organic osmolyte, and expressed on a per embryo basis, as fmol per embryo per minute labeled organic osmolyte (Dawson and Baltz, 1997).

Glycine uptake measurements

Mouse embryos at different stages of preimplantation embryo development (as specified) were rinsed three times in KSOM containing 5 μM ORG23798 or 5 μM DMSO vehicle. Embryos were transferred to culture media containing ^{3}H-glycine plus 5 μM ORG23798 or an equal amount of the vehicle (DMSO) and incubated for 10 minutes at 37°C and 5% CO_2 and then washed as previously described. Radioactivity was measured as counts per minute on a scintillation counter. Using a standard curve, the counts per minute were converted to the number of moles of radiolabeled glycine, and

expressed on a per embryo basis, as fmol per embryo per minute per μM labeled glycine. Specific GLYT1-mediated glycine transport was then calculated by subtracting the rate of transport in the presence of ORG23798 from total transport (in the presence of DMSO vehicle only).

Measuring organic osmolyte accumulation in mouse blastocysts developed from the 8-cell stage as a function of osmolarity (including the effect of ORG23798)

Embryos at the 8-cell stage were isolated from CF1 females 3 days post-hCG. Culture media used were based on KSOM with the addition of 1 μM ^{3}H-organic osmolyte and 1mM of cold organic osmolyte (glutamine, glycine, L-alanine). Embryos were rinsed as stated and placed in microdrops of modified culture media at 250 mOsM, 310 mOsM and 340 and incubated at 37°C and 5% CO_2 under oil in culture dishes for 48 hours and rinsed as previously described. To measure the effect of ORG23798 on glycine and glutamine accumulation, an additional uptake measurement of ^{3}H-glycine and ^{3}H-glutamine was conducted with the addition of 5 μM of ORG23798 or an equal amount of DMSO (vehicle) to the rinse drops and culture media. To show that accumulated ^{3}H-labeled compounds in blastocysts were intracellular rather than in the blastocoele fluid, blastocysts were collapsed prior to washing to determine how much of the total accumulated compound was retained after the blastocoel fluid was released. ^{3}H-organic osmolyte was detected using the scintillation counter and total ^{3}H-organic osmolyte was expressed as fmol per embryo using a standard curve.

Determining the rate of potential organic osmolyte uptake with and without potential competitive inhibitors

Embryos at the morula stage were isolated from CF1 females 3.5 days post-hCG. KSOM at 310 mOsM with the addition of 1 mM of ^{3}H-labeled organic osmolyte (glutamine, glycine, L-alanine or taurine) was used as the culture media. ^{3}H-L-alanine uptake was measured in the presence or absence of 10 mM of established B^{0+} transporter competitive inhibitors (lysine, leucine, or BCH (2-amino-endo-bycyclo[2.2.1]heptane-2-carboxylic acid)). ^{3}H-glycine and ^{3}H-glutamine uptakes were measured in the presence or absence of 10 mM of B^{0+} transporter inhibitors (lysine, leucine, or BCH) or 5 μM of the GLYT1 inhibitor ORG23798 or an equal amount of DMSO. ^{3}H-taurine uptake was measured in the presence or absence of 10 mM of the system β transporter competitive inhibitor β-alanine. Embryos were rinsed and placed in microdrops of modified culture medium and incubated for 10 minutes at 37°C and 5% CO_2 under oil and then washed as previously described. Radioactivity was measured as counts per minute on a scintillation counter and total ^{3}H-organic osmolyte was expressed as fmol per embryo using a standard curve, and then divided by the uptake period to yield the transport rate in fmol per embryo per minute.

Measuring Total Cell Number as a Function of Osmolarity

Mouse blastocysts were stained with Bisbenzimide (Hoechst 33258) using the technique described by Ebert et al. (1985). Blastocysts are placed in acid Tyrode solution (pH 2.5 ± 0.3) for 5-15 seconds until the zona pellucida was removed. Immediately after the zona is removed the embryos were washed through several successive drops of

Hepes-KSOM to neutralize and remove the acid Tyrode. The embryos were then placed in staining media, as described above, and incubated for 30 minutes at 37°C in a warm box. After incubation the embryos were washed quickly through two drops of Hepes-KSOM to remove unbound dye. Embryos were then placed within a well made of transparent tape (Grand&Toy, Don Mills ON) in a small drop of Hepes-KSOM on a clean glass microscope slide. A coverslip was compressed onto the tape well and affixed to the slide using nail polish. Individual nuclei were visualized and counted using a fluorescence microscope with a Hoechst filter set.

Data Analysis

Plots were generated using SigmaPlot 8.2 (SPSS, Chicago, IL). Data were expressed as the mean, and error bars represent the standard error of the mean (SEM). Comparisons between means were made by ANOVA and Tukey-Kramer Multiple Comparison test (InStat, GraphPad Software, San Diego). Proportional data were arcsine-transformed before analysis.

RESULTS

GLYT1 Activity Measurements

Rate of glycine uptake during embryo development

It had previously been shown that GLYT1 is active and functions as an organic osmolyte transporter in 1-cell mouse embryos. However, GLYT1 activity had not been measured at each stage of preimplantation mouse embryo development using the novel specific inhibitor, ORG23798, to conclusively identify the portion of glycine transport activity due to GLYT1. Thus, to elucidate the mechanism of glycine transport at each stage of the mouse preimplantation embryo, ORG23798 was employed. Mouse embryos at the 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst stages were placed in culture media containing radio-labeled glycine for 10 minutes in the presence of 5 μM ORG23798, a concentration previously shown to eliminate virtually all transport by GLYT1 in embryos (Steeves et. al. 2003), or an equal amount of the vehicle DMSO. In the absence of ORG23798, glycine transport was detected at each stage of embryo development (Fig. 4). The highest rates of glycine transport were observed at the 2-cell and blastocyst stage, and the lowest at the 8-cell and morula stages.

In the presence of ORG23798, there was little or no glycine transport in embryos at the 1-cell to the 8-cell stage (Fig. 4). At the morula stage, a small amount of transport that was not inhibited by ORG23798 appeared, but the majority of transport was still inhibited (Fig. 4). In contrast, at the blastocyst stage, a very large rate of glycine transport was evident, but it was not affected by ORG23798 (Fig. 4). Thus, up to the 8-cell stage, all glycine transport is due to GLYT1. At the morula stage, there was little

glycine transport measured, and most was still due to GLYT1 although a component of transport appeared that was resistant to ORG23798. At the blastocyst stage, however, there was very rapid glycine transport that was not due to GLYT1, since it was entirely resistant to ORG23798, indicting the appearance of a different transport mechanism at this stage.

Rate of glycine uptake via GLYT1

To determine the rate of glycine uptake via GLYT1, the total rate of glycine uptake (measured in DMSO vehicle alone) less the rate of glycine uptake in the presence of ORG23798 was calculated (Fig. 5). GLYT1 was active from the 1-cell through 8-cell stages, decreased from the maximal levels at the 1-cell to 4-cell stages to a very small amount at the morula stage, and was absent from blastocysts. Therefore, GLYT1 becomes inactive around the time of compaction. This indicates that any accumulation of glycine as an organic osmolyte after compaction cannot be via GLYT1 and must be via a different transport system.

Figure 4: Effect of ORG23798 on the accumulation of glycine during embryo development.

Rate of uptake was determined from the total glycine accumulated in 10 min in the presence of 10 μM [^{3}H]glycine with 5μM ORG23798 or 10 μM [^{3}H]glycine with 5μM DMSO. Each point represents data from 24 – 50 embryos in 3-5 replicates. The error bars are SEMs of the replicates.

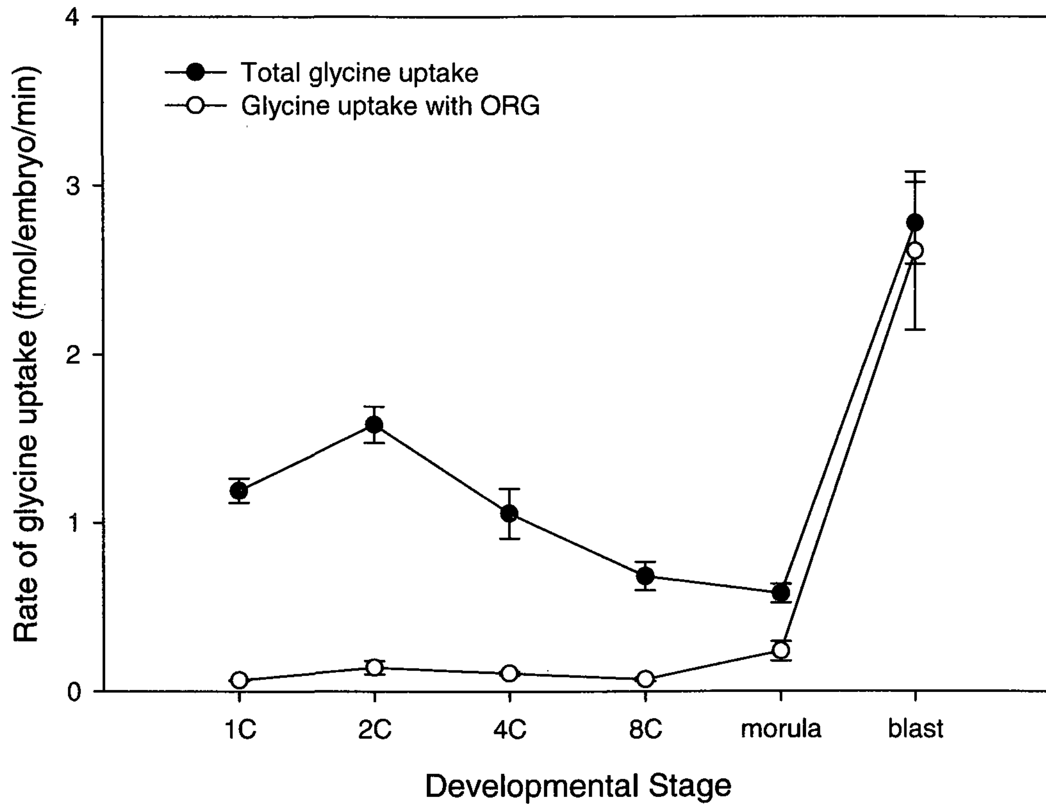
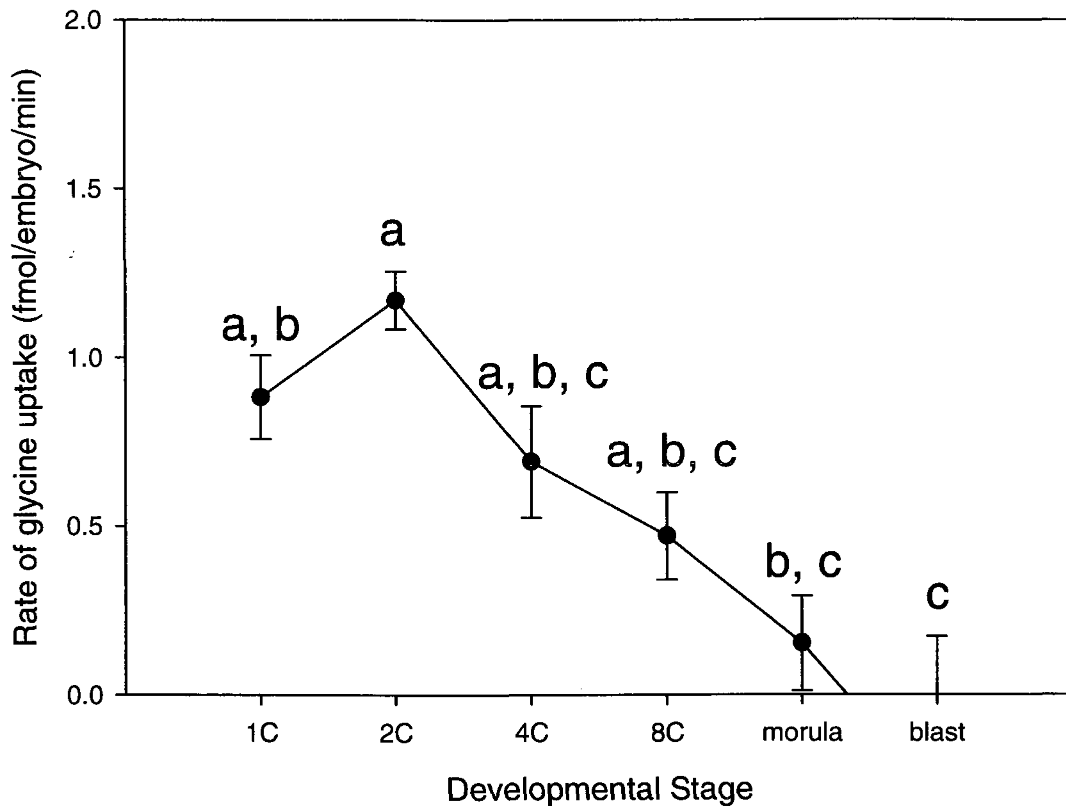


Figure 5: Rate of glycine uptake via the glycine specific transporter GLYT1 during embryo development.

The rate of glycine accumulation in the absence of ORG23798 (5 μM DMSO) less the rate of glycine accumulation in the presence of ORG23798. Original data are shown in Fig 4. Points that do not share the same letter are significantly different ($P<0.05$). Each point represents data from 24 – 50 embryos in 3-5 replicates. The error bars are SEMs of the replicates.

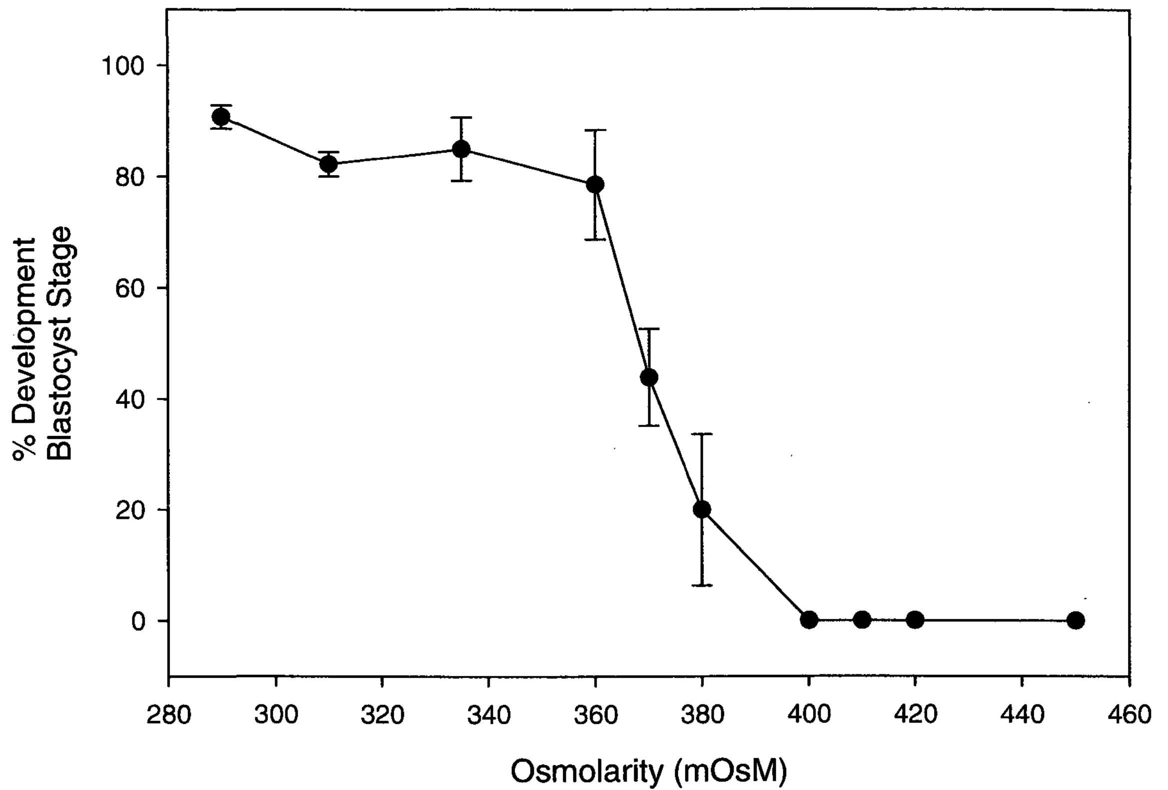


Effect of Osmolarity on Post-Compaction Embryo Development

The viability of compaction-stage embryos cultured in media as a function of increased osmolarity was determined to study the dependence of post-compaction embryo development on external osmolarity. The ability of compaction-stage embryos to develop at high osmolarities was determined using culture media at 250, 310, 335, 360, 370, 380, 400, 410, 420 and 450 mOsM. Eight-cell mouse embryos were placed into culture media on day 1 of culture and were scored as blastocysts if a blastocoel cavity was visible, regardless of size, on day 3. Eight-cell mouse embryos developed into blastocysts by day 3 in culture media that were between 250 and 360 mOsM (Fig. 6). The percentage of 8-cell embryos that reached the blastocyst stage successively decreased in culture media at 360 to 450 mOsM (Fig. 6). In culture media with an osmolarity of 400 mOsM, 8-cell mouse embryos are able to form into morula, however blastocysts could not develop at 400 mOsM. Thus an osmolarity of 400 mOsM completely prevents compaction-stage embryo development to blastocysts, while most embryos were able to form blastocysts at osmolarities up to 360 mOsM.

Figure 6: Effect of increasing osmolarity on embryo development to the blastocyst stage.

8-cell mouse embryos were cultured in modified KSOM at varying osmolarities. Each point represents the mean ± SEM of at least 3 repeats. Error bars represent the SEMs of the repeats.



Effect of Potential Organic Osmolytes on Post-Compaction Stage Embryos

To determine whether potential organic osmolytes could rescue embryo development from the 8-cell to blastocyst stages at high osmolarity, several potential organic osmolytes were tested to determine their effectiveness at alleviating the detrimental effects of high osmolarity in compaction-stage embryos. To determine the osmoprotective abilities of different organic osmolytes, compaction-stage mouse embryo development was determined in culture media at 400 mOsM in the absence or presence of 1 mM of potential organic osmolytes (Table I). A concentration of 1 mM of each organic osmolyte was chosen, as this concentration has previously been shown to be effective at providing osmoprotection to cleavage-stage embryo development (Dawson and Baltz, 1997) and is likely to saturate any transport system present. A culture medium osmolarity of 400 mOsM was chosen because, as described above, 8-cell mouse embryos cannot develop to the blastocyst stage at this osmolarity, but at least some will develop at lower osmolarities. Therefore, any blastocyst development at 400 mOsM in the presence of a possible organic osmolyte was taken to be an indication of its possible ability to support embryo development at increased osmolarity, and led to its selection for further testing. There was a significant difference in blastocyst development in the presence of 1 mM of glutamine ($p < 0.0001$; ANOVA), glycine ($p < 0.0001$; ANOVA), or L-alanine ($p < 0.0001$; ANOVA), compared to development without the addition of organic osmolyte (Fig. 7 –9).

β-Alanine at a concentration of 1 mM was not significantly effective ($P > 0.05$; ANOVA) at sustaining development to the blastocyst stage, but there was a trend towards some development (Fig. 10). Therefore a concentration of 5 mM β-alanine was tested, as

this dose has previously been shown to confer maximal protective effects to 1-cell mouse embryos against the deleterious effect of increasing osmolarity (Dawson and Baltz, 1997). At 5 mM, β-alanine exerted a significant protective effect on development to the blastocyst stage ($p < 0.0001$; ANOVA) (Fig 11).

In contrast betaine, proline, taurine and myo-inositol were entirely ineffective at supporting 8-cell mouse embryo development to the blastocyst stage (Fig 12-15). For betaine and proline, this is in contrast to their effectiveness in protecting 1-cell embryo development at higher osmolarity (Dawson and Baltz, 1997).

Figure 7: Effect of glutamine on 8-cell mouse embryo development to the blastocyst stage at high osmolarity

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM with and without 1 mM of glutamine and in 250 mOsM KSOM as a control. Each bar represents the mean ± SEM of 60-75 embryos cultured in at least five replicates. ANOVAs were done on each day of development. No comparison was done between days. Asterisks indicate a significant difference between a treatment group and the group with no addition (***p < 0.0001; *p < 0.05).

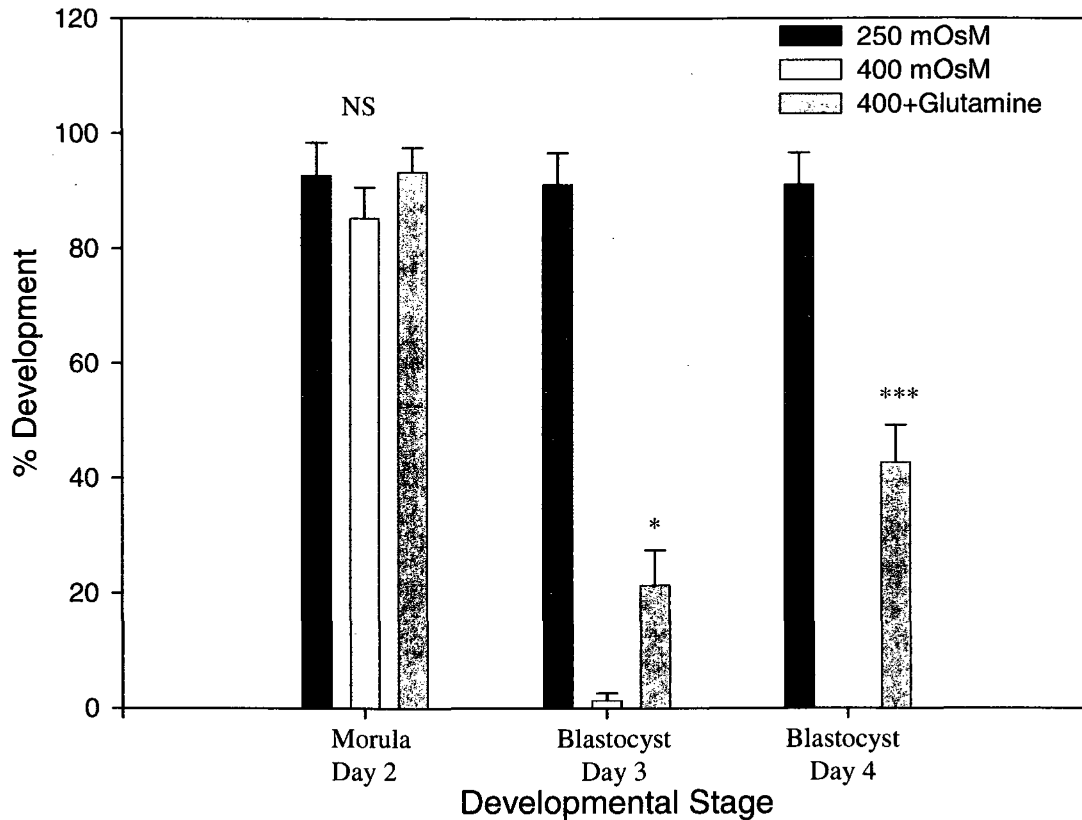


Figure 8: Effect of glycine on 8-cell mouse embryo development to the blastocyst stage at high osmolarity

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM with and without 1 mM of glycine and in 250 mOsM KSOM as a control. Each bar represents the mean ± SEM of 54-75 embryos cultured in at least five replicates. ANOVAs were done on each day of development. No comparison was done between days. Asterisks indicate a significant difference between a treatment group and the group with no addition (***$p < 0.0001$).

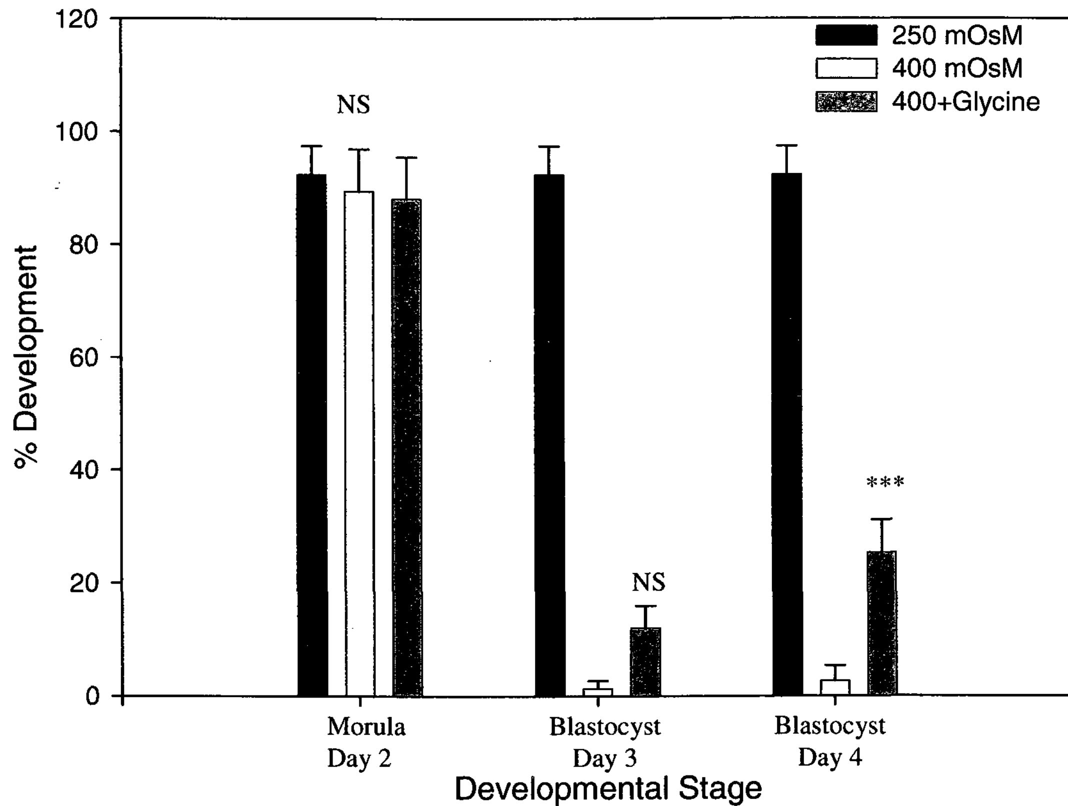


Figure 9: Effect of L-alanine on 8-cell mouse embryo development to the blastocyst stage at high osmolarity

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM with and without 1 mM of L-alanine and in 250 mOsM KSOM as a control. Each bar represents the mean ± SEM of 57-75 embryos cultured in at least five replicates. ANOVAs were done on each day of development. No comparison was done between days. Asterisks indicate a significant difference between a treatment group and the group with no addition (***p < 0.0001, *p < 0.05).

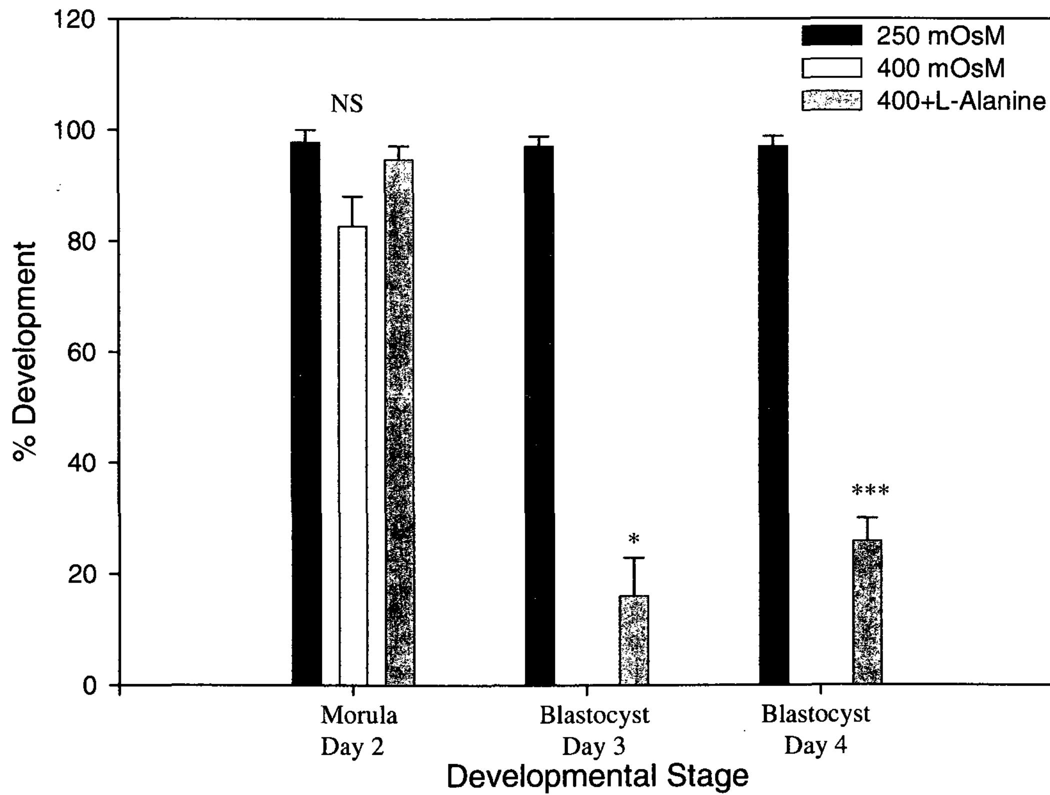


Figure 10: Effect of β-alanine on 8-cell mouse embryo development to the blastocyst stage at high osmolarity

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM with and without 1 mM of β-alanine and in 250 mOsM KSOM as a control. Each bar represents the mean ± SEM of 39-75 embryos cultured in at least five replicates. ANOVAs were done on each day of development. No comparison was done between days. There is no significant difference at the blastocyst stage on day 2 or day 3 ($p > 0.05$).

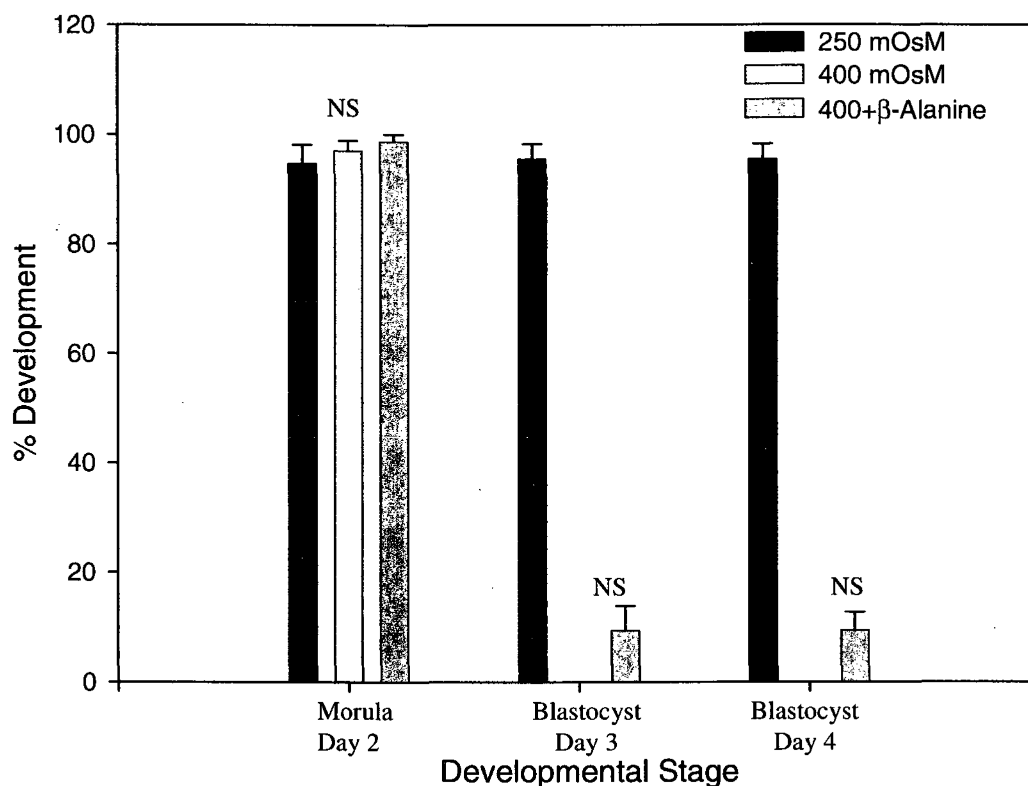


Figure 11: Effect of β-alanine on 8-cell mouse embryo development to the blastocyst stage at high osmolarity

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM with the addition of 1 mM of β-alanine or 5 mM or β-alanine or without and in 250 mOsM KSOM as a control. Each bar represents the mean ± SEM of 23-45 embryos cultured in at least three replicates. ANOVAs were done on each day of development. No comparison was done between days. Asterisks indicate a significant difference between a treatment group and the group with no addition (***p < 0.0001, *p < 0.05).

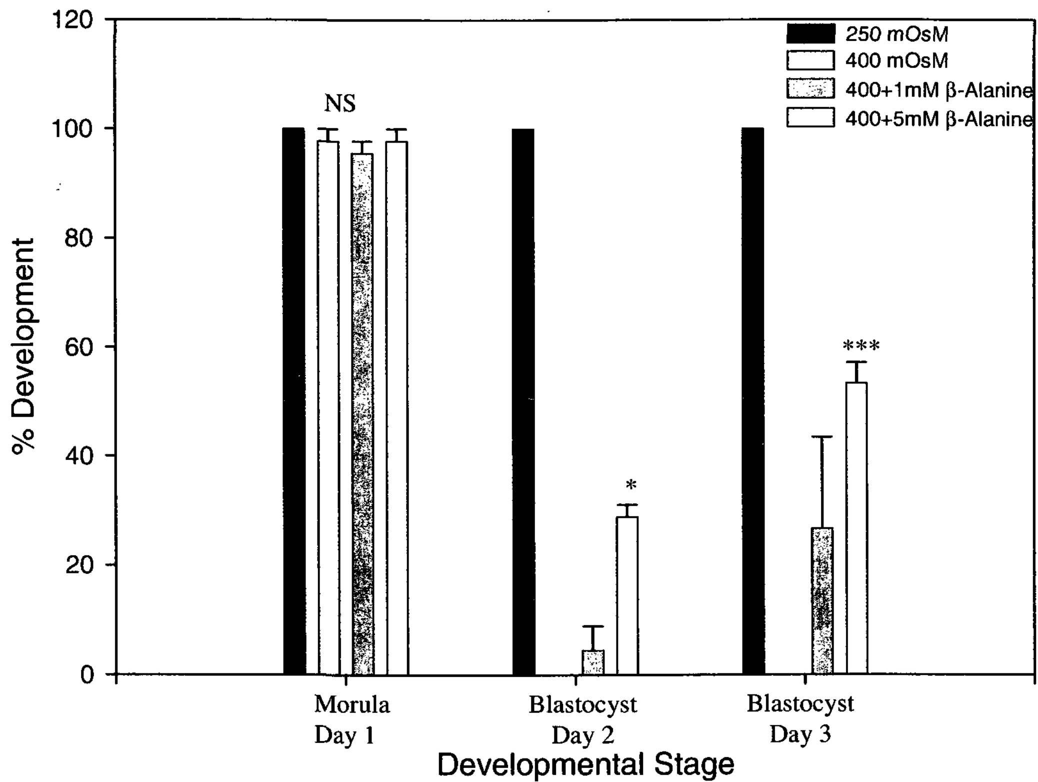


Figure 12: Effect of betaine on 8-cell mouse embryo development to the blastocyst stage at high osmolarity

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM with and without 1 mM of betaine and in 250 mOsM KSOM as a control. Each bar represents the mean ± SEM of 46-74 embryos cultured in at least five replicates. ANOVAs were done on each day of development. No comparison was done between days. There is no significant difference at the blastocyst stage on day 3 or day 4 ($p > 0.05$).

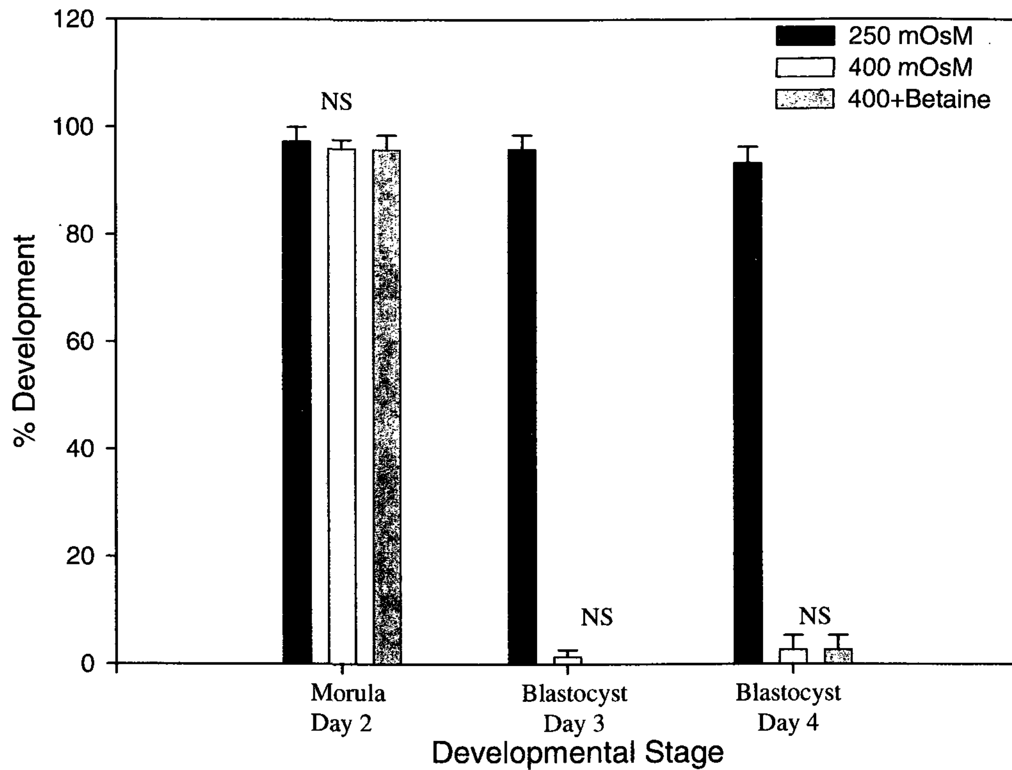


Figure 13: **Effect of proline on 8-cell mouse embryo development to the blastocyst stage at high osmolarity**

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM with and without 1 mM of proline and in 250 mOsM KSOM as a control. Each bar represents the mean ± SEM of 67-75 embryos cultured in at least five replicates. ANOVAs were done on each day of development. No comparison was done between days. There is no significant difference at the blastocyst stage on day 3 or day 4 ($p > 0.05$).

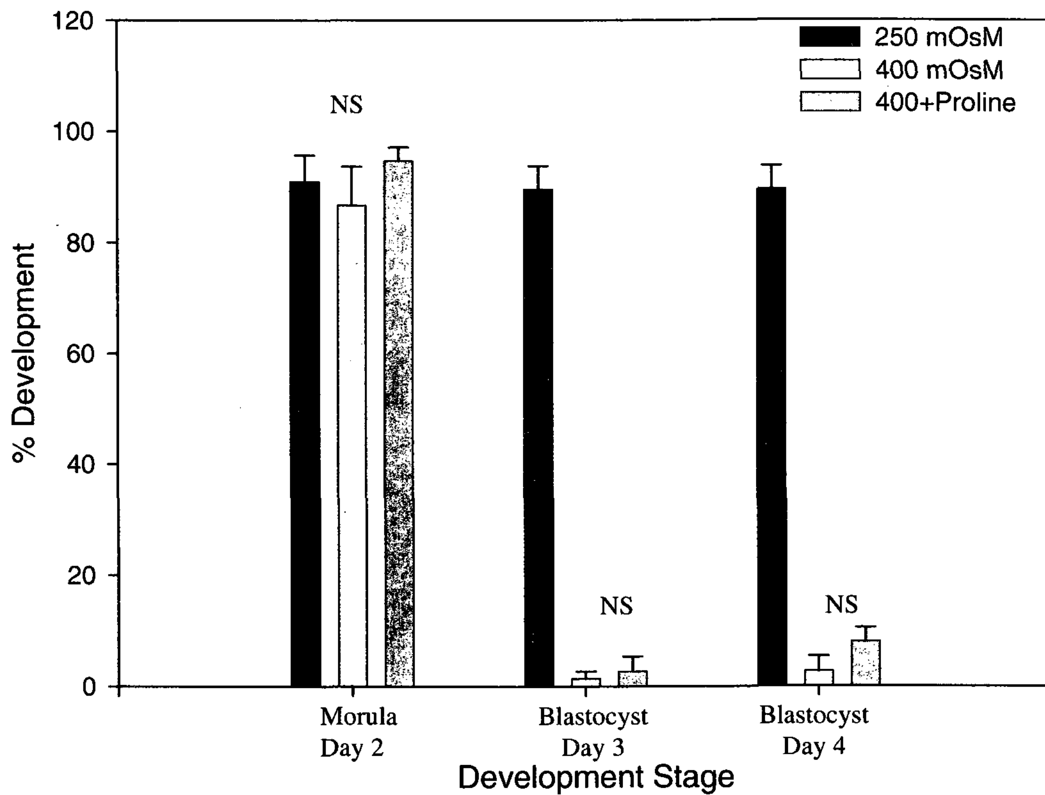


Figure 14: Effect of taurine on 8-cell mouse embryo development to the blastocyst stage at high osmolarity

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM with and without 1 mM of taurine and in 250 mOsM KSOM as a control. Each bar represents the mean ± SEM of 67-75 embryos cultured in at least five replicates. ANOVAs were done on each day of development. No comparison was done between days. There is no significant difference at the blastocyst stage on day 3 or day 4 ($p > 0.05$).

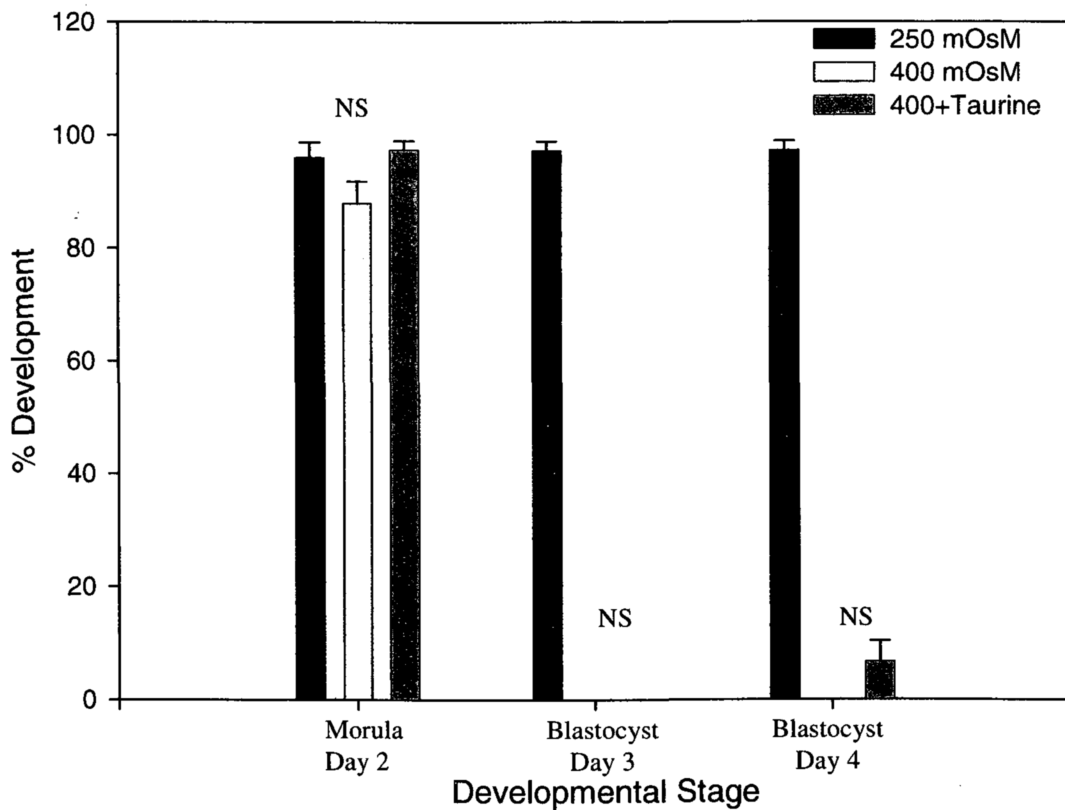
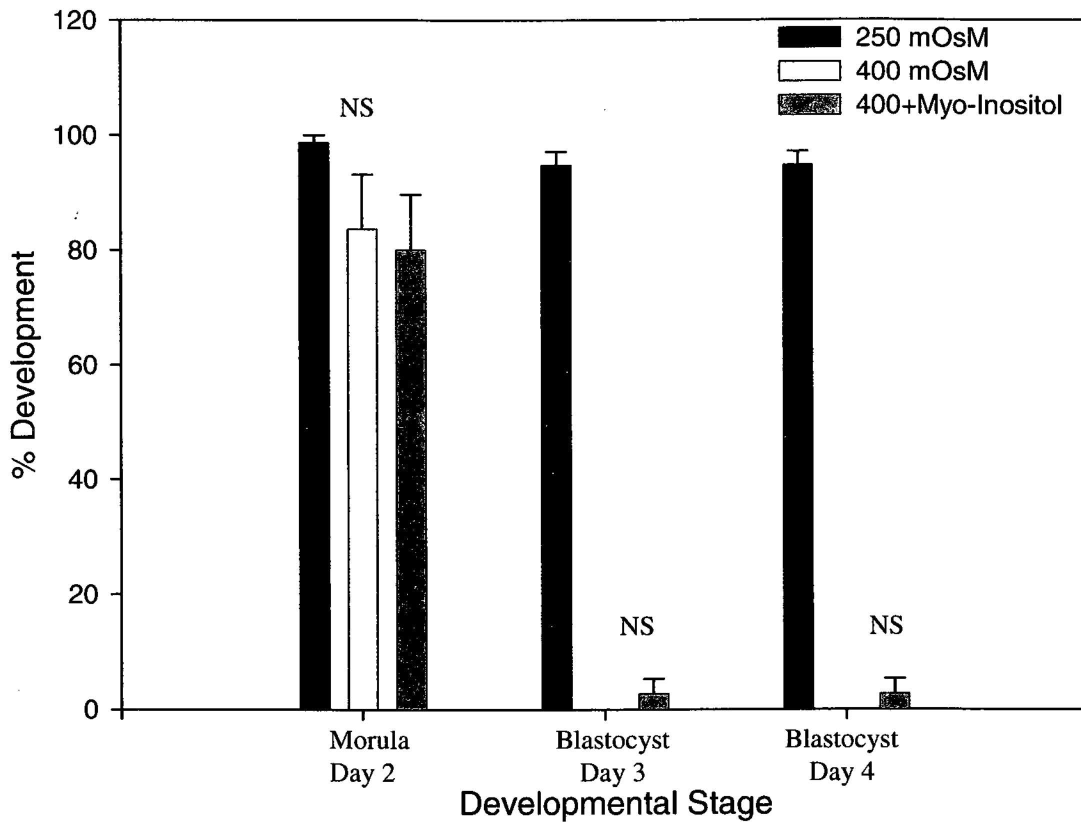


Figure 15: Effect of myo-inositol on 8-cell mouse embryo development to the blastocyst stage at high osmolarity

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM with and without 1 mM of myo-inositol and in 250 mOsM KSOM as a control. Each bar represents the mean ± SEM of 56-75 embryos cultured in at least five replicates. ANOVAs were done on each day of development. No comparison was done between days. There is no significant difference at the blastocyst stage on day 3 or day 4 ($p > 0.05$).



Effect of Organic Osmolytes on Post-Compaction Stage Embryo Development

Potential organic osmolytes were tested to determine their ability to protect mouse embryos at different osmolarities of culture medium. The four organic osmolytes: glutamine, glycine, L-alanine and β-alanine, identified to be effective at alleviating the detrimental effects of high osmolarity on compaction-stage embryo development, were tested to determine their effect in culture media with osmolarities at 250, 310, 340, 370 and again at 400 mOsM. At 250 and 310 mOsM there was no significant difference in blastocyst development with the addition of organic osmolyte than without (Fig.16-17), although there was a trend towards significance on day 3 at 250 mOsM, probably reflecting the small decrease in development seen with β-alanine. At 340 mOsM a significant improvement was observed in blastocyst development with the addition of each organic osmolyte than without on day 4. The addition of any of the four potential organic osmolytes was able to increase blastocyst development from about 60% to 90-100% (Fig. 18). The addition of organic osmolyte at 370 mOsM significantly increased blastocyst development on day 4. Glutamine and β-alanine increased blastocyst development from approximately 10% to 80-90% (Fig. 19). At 400 mOsM, in the absence of any organic osmolyte, 8-cell mouse embryos were unable to develop into blastocysts, but development was significantly rescued on day 4 by glutamine or β-alanine, while glycine and L-alanine did not have an effect at 400 mOsM in this set of experiments.

Taken together, these results showed that glutamine, glycine, L-alanine or β-alanine were effective at protecting development of most 8-cell embryos to blastocysts at

osmolarities up to 370 mOsM, and that the maximal improvement in development was seen at 370 mOsM.

Figure 16: Effect of organic osmolytes on 8-cell mouse embryo development to the blastocyst stage in 250 mOsM KSOM

8-cell mouse embryos were cultured in modified KSOM at 250 mOsM in the presence and absence organic osmolyte (1 mM of glutamine, glycine or L-alanine or 5 mM or β-alanine). Each bar represents the mean ± SEM 42-60 embryos cultured in at least three replicates. ANOVAs were done at each day of development. No comparison was done between days. There is no significant difference at the blastocyst stage on day 3 (p=0.0895) or day 4 (p = 0.0610).

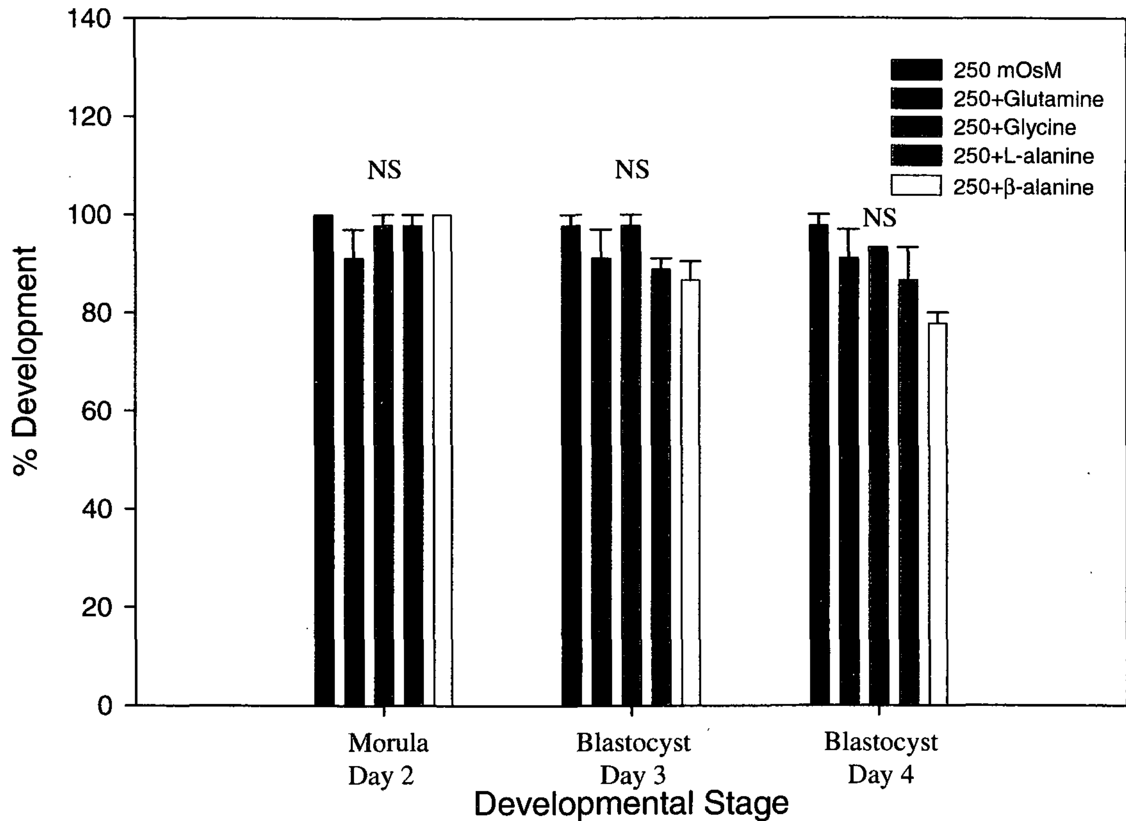


Figure 17: Effect of organic osmolytes on 8-cell mouse embryo development to the blastocyst stage in 310 mOsM KSOM

8-cell mouse embryos were cultured in modified KSOM at 310 mOsM in the presence and absence organic osmolyte (1 mM of glutamine, glycine or L-alanine or 5 mM or β-alanine). Each bar represents the mean ± SEM of 40-45 embryos cultured in at least three replicates. ANOVAs were done at each day of development. No comparison was done between days. There is no significant difference at the blastocyst stage on day 3 (p=0.0818) or day 4 (p = 0.4355).

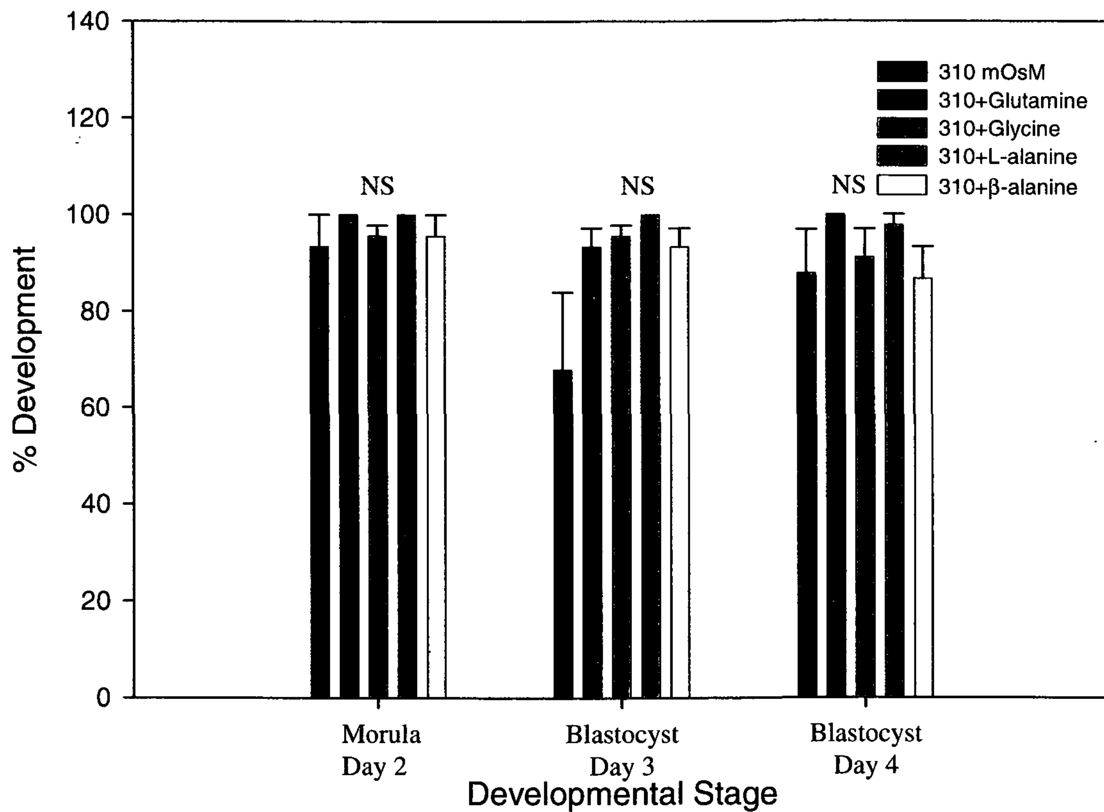


Figure 18: Effect of organic osmolytes on 8-cell mouse embryo development to the blastocyst stage in 340 mOsM KSOM

8-cell mouse embryos were cultured in modified KSOM at 340 mOsM in the presence and absence organic osmolyte (1 mM of glutamine, glycine or L-alanine or 5 mM or β-alanine). Each bar represents the mean ± SEM of 43-45 embryos cultured in at least three replicates. ANOVAs were done at each day of development. No comparison was done between days. Asterisks indicate a significant difference between a treatment group and the group with no addition (*p < 0.05, **p < 0.01).

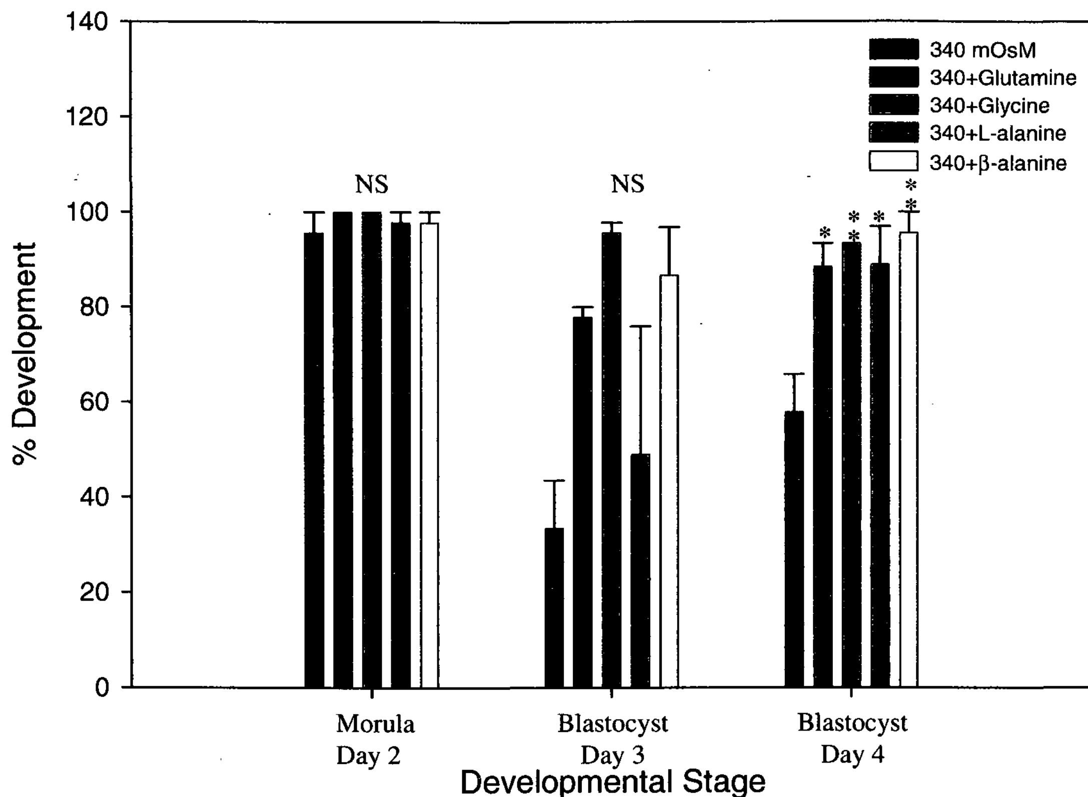


Figure 19: Effect of organic osmolytes on 8-cell mouse embryo development to the blastocyst stage in 370 mOsM KSOM

8-cell mouse embryos were cultured in modified KSOM at 370 mOsM in the presence and absence organic osmolyte (1 mM of glutamine, glycine or L-alanine or 5 mM or β-alanine). Each bar represents the mean ± SEM of 38-45 embryos cultured in at least three replicates. ANOVAs were done at each day of development. No comparison was done between days. Asterisks indicate a significant difference between a treatment group and the group with no addition ($^{c}p < 0.001$).

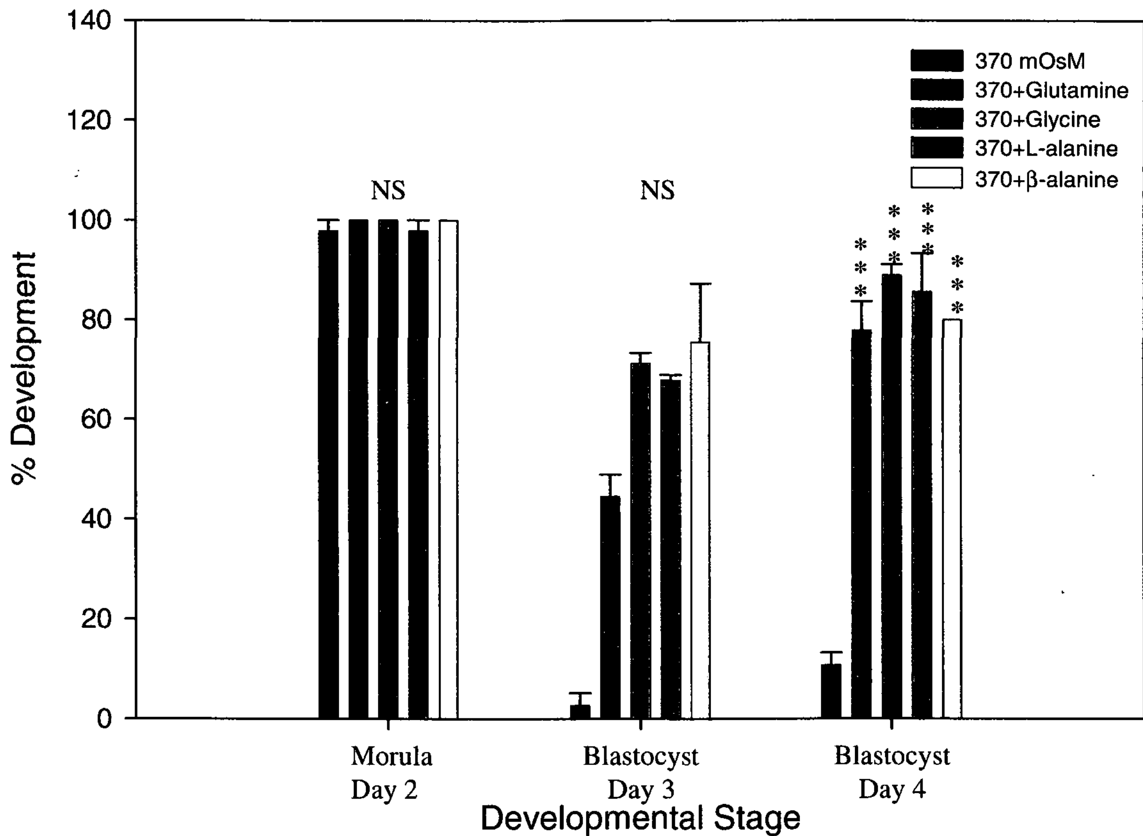
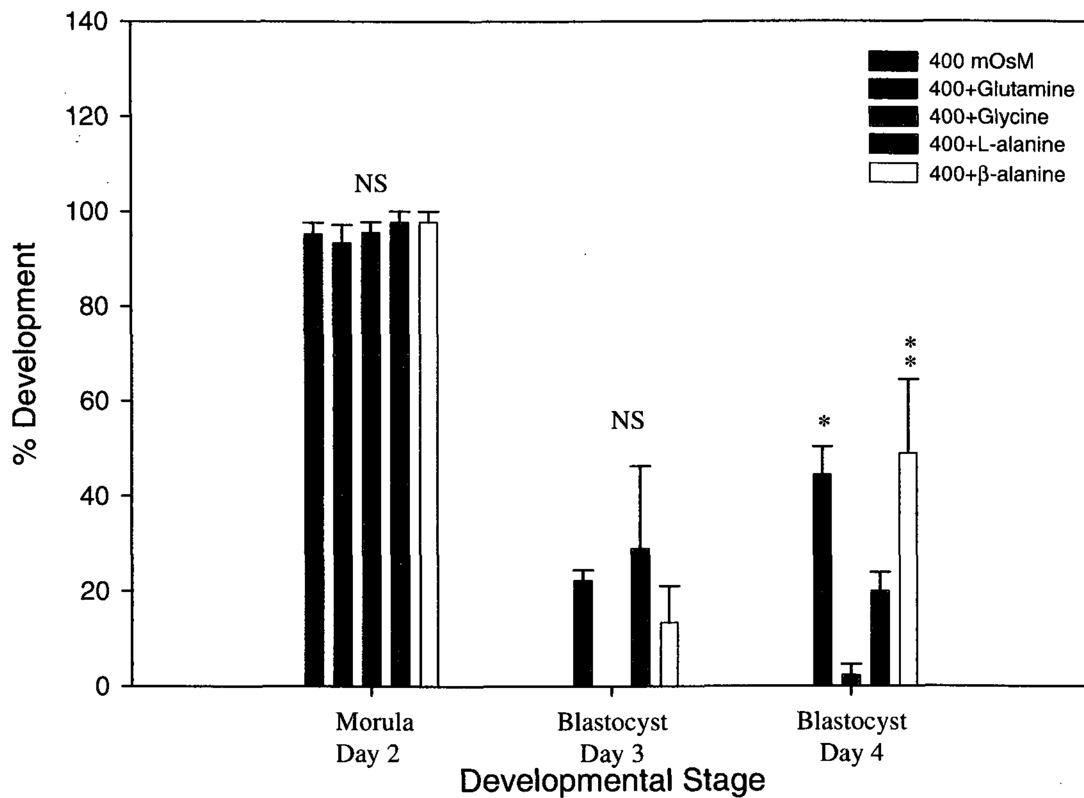


Figure 20: Effect of organic osmolytes on 8-cell mouse embryo development to the blastocyst stage in 400 mOsM KSOM

8-cell mouse embryos were cultured in modified KSOM at 400 mOsM in the presence and absence organic osmolyte (1 mM of glutamine, glycine or L-alanine or 5 mM or β-alanine) and in 250 mOsM KSOM as a control. Each point represents the mean ± SEM of 36-45 embryos cultured in at least three replicates. ANOVAs were done at each day of development. No comparison was done between days. Asterisks indicate a significant difference between a treatment group and the group with no addition (*p < 0.05, **p < 0.01).



Organic Osmolyte Accumulation During 48 hours (8-cell to blastocyst stage) as a Function of External Osmolarity

Three of the organic osmolytes identified in post-compaction stage embryos, glutamine, glycine and L-alanine, were tested to determine whether organic osmolyte accumulation is increased with increasing osmolarity, as had been found for glycine and betaine in 1-cell embryos cultured to the 2-cell stage (Anas et al., 2007; Steeves et al., 2003). β-alanine was not tested because it was not available commercially in a ^{3}H-labelled form. Eight-cell mouse embryos were cultured in 250 KSOM or in KSOM with the osmolarity raised to 310 or 340 mOsM using raffinose. The media contained 1 mM total of one of the organic osmolytes (1:1000 [^{3}H]-labelled organic osmolyte:organic osmolyte). On day 3 of culture, when the 8-cell embryos had developed into blastocysts, the total accumulation of each of the organic osmolyte was measured. To ensure that the accumulated compound was intracellular rather than in the blastocoele fluid, blastocysts were collapsed prior to washing. No significant increase in intracellular organic osmolyte accumulation was observed between the different osmolarities (Fig 21-23). Therefore it appears that organic osmolyte accumulation is not affected by increasing osmolarity, unlike in cleavage-stage embryos.

Figure 21: Glutamine accumulation as a function of osmolarity

8-cell mouse embryos were cultured in modified KSOM at 250, 310 and 340 mOsM to the blastocyst stage with the addition of 1 μM of [^{3}H]glutamine and 1 mM glutamine. Each bar represents data from 27-34 embryos in five replicates. The error bars are SEMs of the replicates. ANOVAs were done at each day of development. There was no significant difference (p=0.0757)

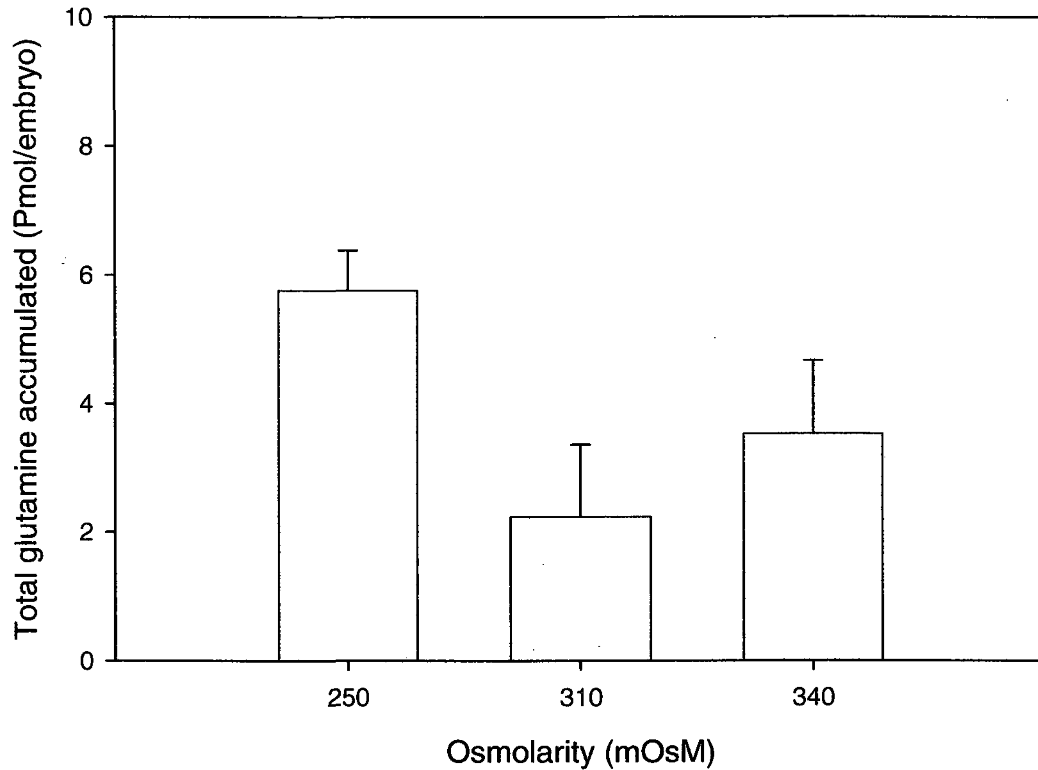


Figure 22: Glycine accumulation as a function of osmolarity

8-cell mouse embryos were cultured in modified KSOM at 250, 310 and 340 mOsM with the addition of 1 μM of [^{3}H]glycine and 1 mM glycine. Each bar represents data from 34-41 embryos in five replicates. The error bars are SEMs of the replicates. ANOVAs were done at each day of development. There was no significant difference (p=0.0541)

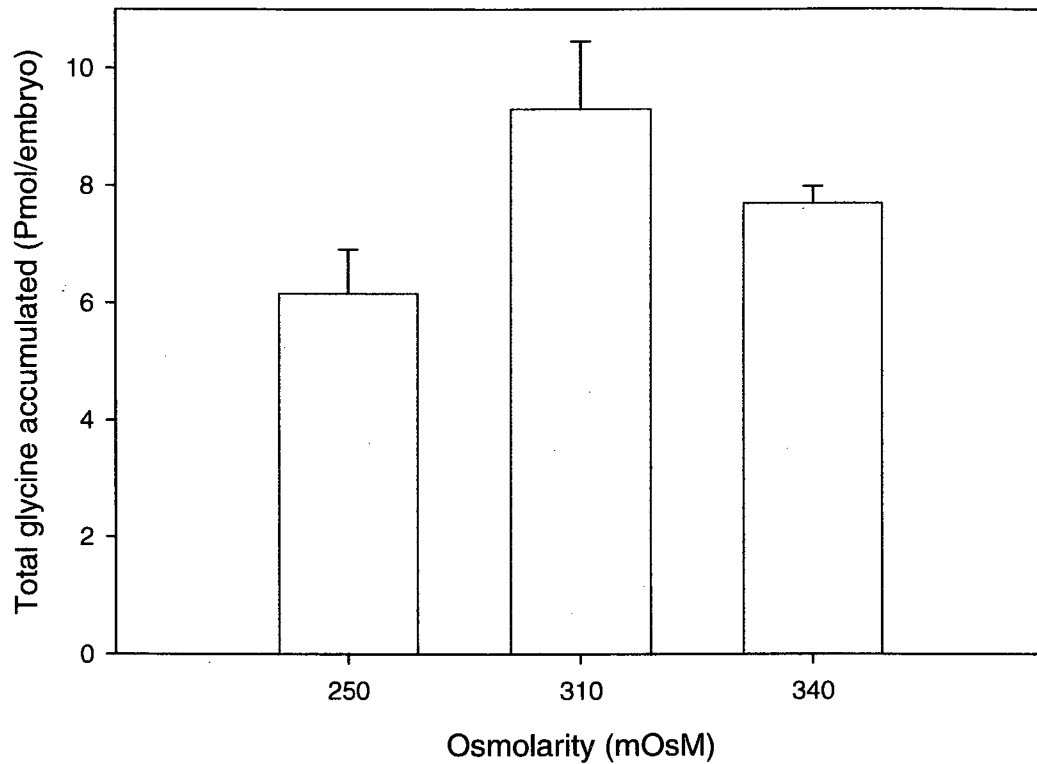
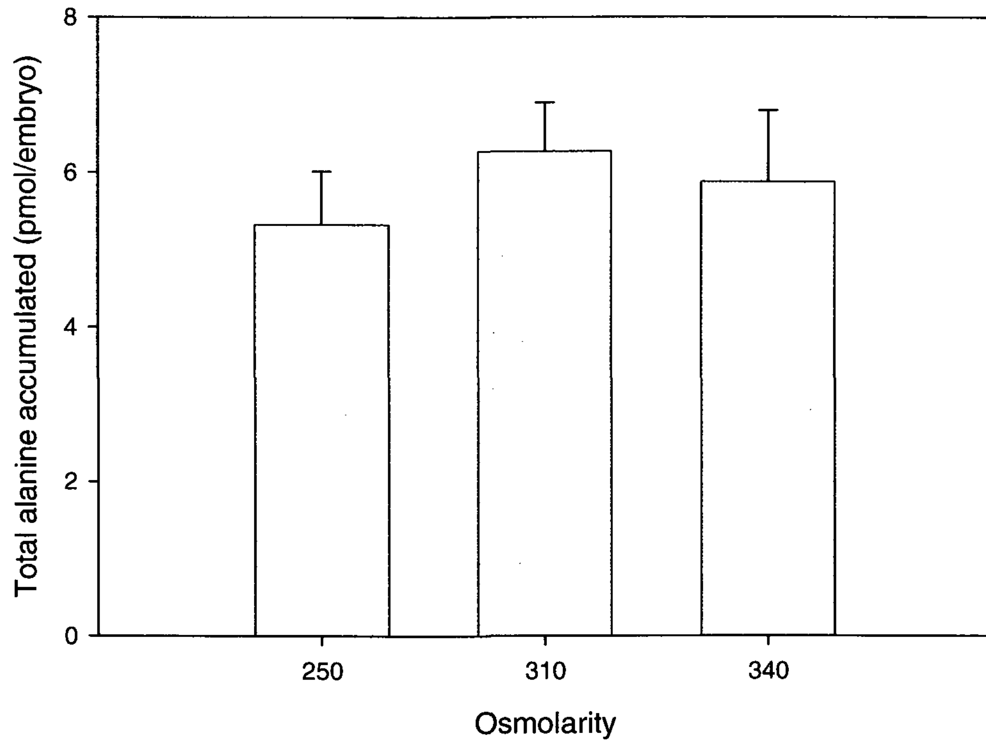


Figure 23: L-Alanine accumulation as a function of osmolarity

8-cell mouse embryos were cultured in modified KSOM at 250, 310 and 340 mOsM with the addition of 1 μM of [^{3}H]L-alanine and 1 mM L-alanine. Each bar represents data from 33-42 embryos in five replicates. The error bars are SEMs of the replicates. ANOVAs were done at each day of development. There was no significant difference (p=0.6786)



Effect of Organic Osmolytes on Blastocyst Volume

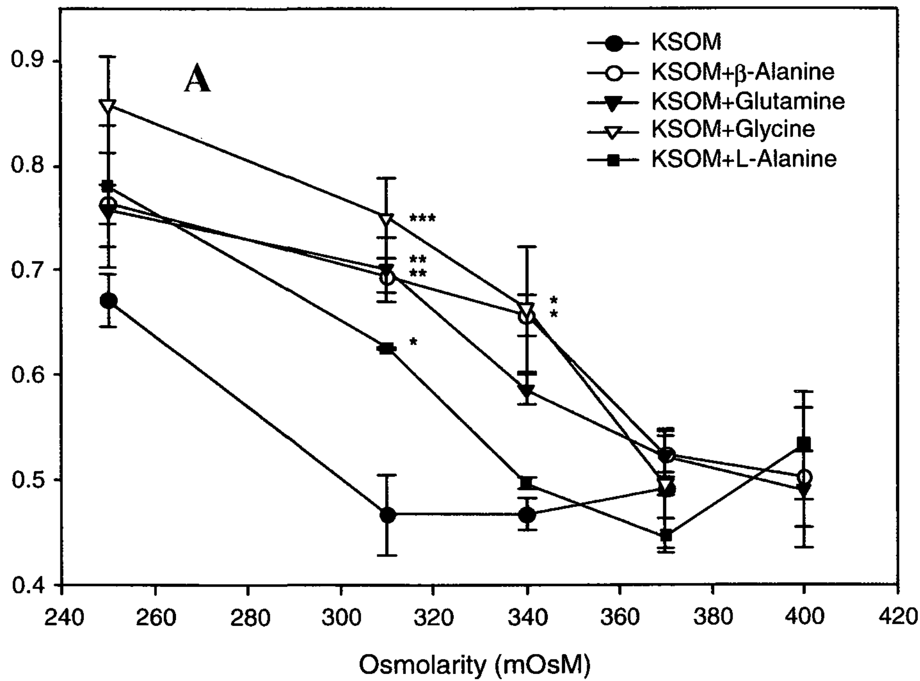
One key function of blastocysts is to transport fluid across the trophectoderm into the blastocoel and thus cause blastocyst expansion. It is possible that increased osmolarity could cause a decrease in blastocyst size because of impaired expansion, particularly given that most of the measured volume of an expanded blastocyst consists of blastocoel. Alternatively, increased osmolarity could cause a decrease in cell proliferation, which also likely would result in decreased overall size. Therefore, we determined whether each of the four osmoprotective amino acids had an effect on blastocyst size at different osmolarities. Eight-cell mouse embryos were cultured in media at 250, 310, 340, 370 and 400 mOsM in the absence or presence of 1 mM glutamine, glycine or L-alanine, or of 5 mM of β-alanine, and the volume of each blastocyst measured on day 3 and day 4 of culture (Fig. 3).

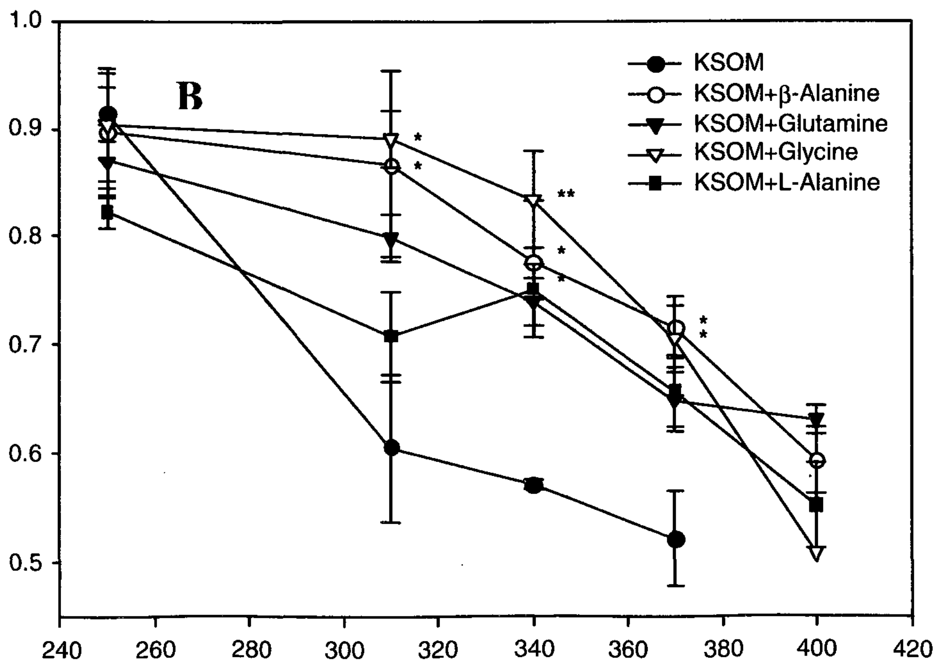
There was no significant difference in blastocyst volume in culture media at 250 mOsM on day 3 and day 4 of culture with the addition of organic osmolyte compared to without (Fig 24). Embryos cultured at 310 mOsM had an approximately 30% greater volume with the addition of glutamine ($p < 0.001$; ANOVA), glycine ($p < 0.001$; ANOVA) or β-alanine ($p < 0.001$; ANOVA) than without on day 3 of culture. Similarly in the presence of L-alanine blastocysts had an approximately 25% greater volume ($p < 0.05$; ANOVA) than in its absence on day 3 of culture. (Fig 24A). On day 4 of culture, embryos cultured at 340 mOsM had on average approximately 30% greater volumes with the addition of β-alanine ($p < 0.05$; ANOVA) and glutamine ($p < 0.05$; ANOVA) than without, and an approximately 25% greater mean volume in the presence of glycine ($p < 0.01$; ANOVA) than in its absence (Fig 24B).

At 370 mOsM, blastocysts had a greater volume on day 4 of culture with the addition of organic osmolyte than without. The presence of glutamine and L-alanine resulted in an approximately 20% greater blastocyst volume, while embryos cultured in presence of glycine had an approximately 25% greater volume. Embryos had an approximately 30% greater volume on day 4 of culture, a significant increase, in the presence of β-alanine ($p < 0.05$; ANOVA) (Fig. 24B). Thus, the presence of each of the organic osmolytes up to 340 mOsM resulted in a trend toward higher volume (except for L-alanine on day 3).

Figure 24: Effect of organic osmolytes on blastocyst volume

8-cell mouse embryos were cultured in modified KSOM at 250, 310, 340, 370 and 400 mOsM with and without organic osmolyte (1 mM glutamine, glycine or L-alanine or 5 mM β-alanine). Blastocyst volume was calculated from blastocyst measurement on day 3 (A) and day 4 (B) of culture. Each point represents data from 36-45 embryos cultured in at least 3 replicates. The error bars are SEMs of the replicates. ANOVAs were preformed at each osmolarity. Asterisks indicate a significant difference between a treatment group and the group with no addition. No analysis was preformed at 400 mOsM, because no blastocysts developed in some groups.





Effect of the Identified Organic Osmolytes on Cell Number in Blastocysts

The ability of an organic osmolytes to maintain blastocyst volume may be due at least in part to a capability of organic osmolytes to support a greater blastocyst cell number. To test this prediction, mouse blastocysts were stained with Hoechst 33258, a DNA-binding dye on day 3, after being cultured from the 8-cell stage in media at 310 mOsM and 370 mOsM with or without 1 mM of glutamine, glycine or L-alanine, or 5 mM of β-alanine. A medium osmolarity of 310 mOsM was chosen since 310 mOsM is close to the in vivo osmolarity of mouse oviductal fluid (Collins and Baltz, 1999) and uterine fluid, and a significant increase in blastocyst volume was observed at this osmolarity in the presence of organic osmolyte compared to without any addition (above). A medium osmolarity of 370 mOsM was also chosen, as it is the highest osmolarity at which a significant number of 8-cell mouse embryos could develop into blastocysts without the addition of organic osmolyte. In addition, the presence of organic osmolytes resulted in greater blastocyst volumes in embryos cultured from the 8-cell stage at 370 mOsM.

There was no significant difference in cell number observed in blastocyst developed in media at 310 mOsM in the presence of organic osmolyte compared to without addition (Fig 25) ($p > 0.05$; ANOVA). At 370 mOsM, however, a significant difference was observed in blastocyst cell number in the presence of the four organic osmolytes, glutamine ($p < 0.001$), glycine ($p < 0.001$), L-alanine ($p < 0.001$), or β-alanine ($p < 0.001$), than without (Fig 26).

Figure 25: Effect of organic osmolytes on total mouse blastocyst cell number 8-cell embryos developed to the blastocyst stage in 310 mOsM KSOM

8-cell mouse embryos were cultured in modified KSOM 310 in the presence and absence of organic osmolyte (1 mM glutamine, glycine or L-alanine or 5 mM β-alanine) to the blastocyst stage. Individual cells of the blastocysts were visualized using a DNA-binding dye and counted. Each bar represents the mean ± SEM of 15-20 embryos in 3-4 replicates. There was no significant difference in cell number in the presence and absence of organic osmolyte ($p>0.05$; by ANOVA)

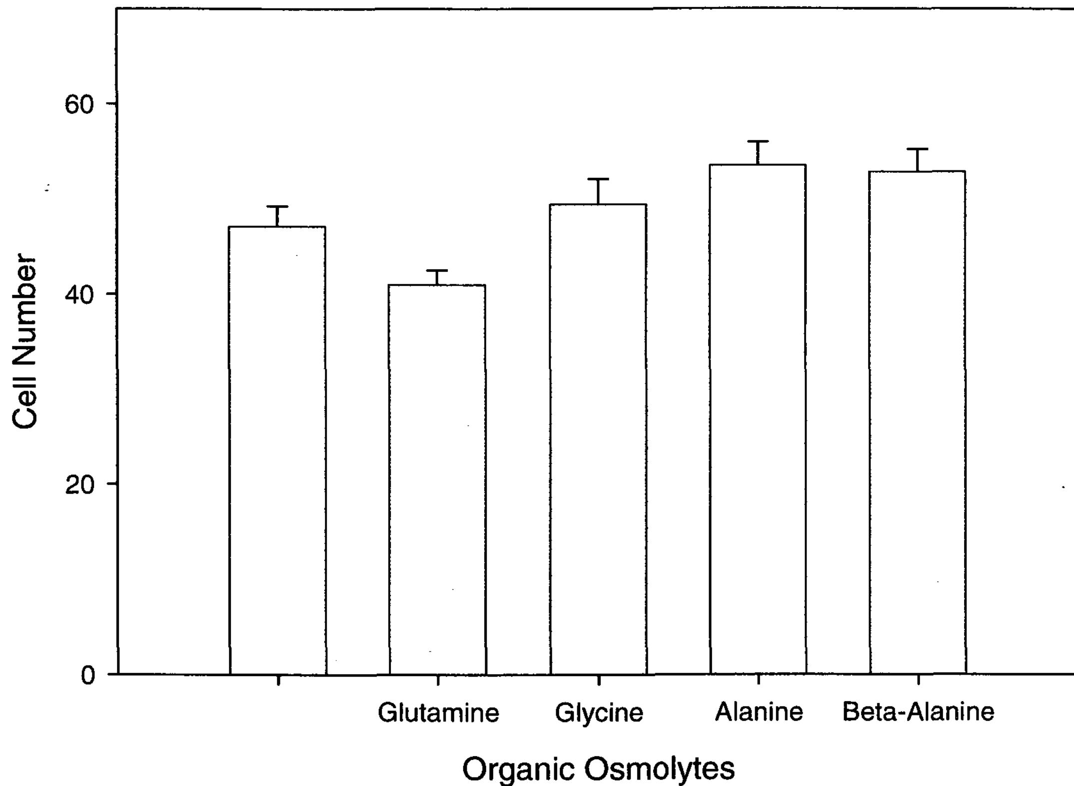
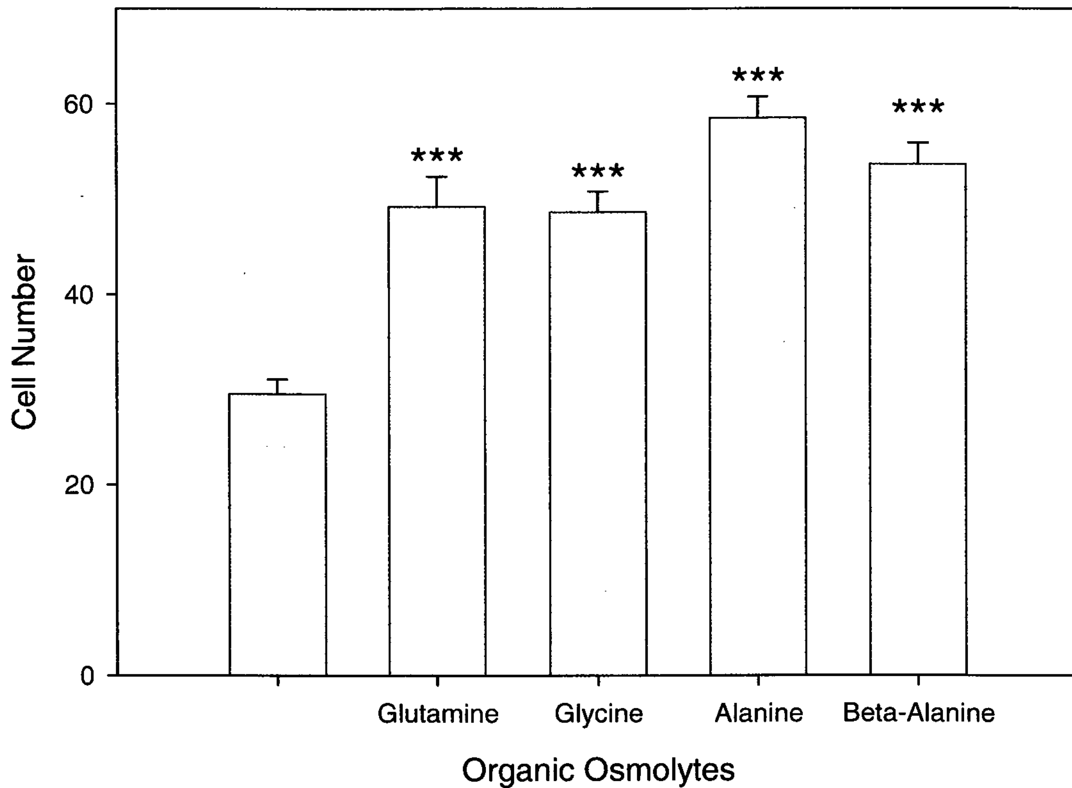


Figure 26: Effect of organic osmolytes on total mouse blastocyst cell number 8-cell embryos developed to the blastocyst stage in 370 mOsM KSOM

8-cell mouse embryos were cultured in modified KSOM 370 in the presence and absence of organic osmolyte (1 mM glutamine, glycine or L-alanine or 5 mM β-alanine) to the blastocyst stage. Individual cells of the blastocysts were visualized using a DNA-binding dye and counted. Each bar represents the mean ± SEM of at least 15 embryos in three replicates. Asterisks indicate a significant difference between a treatment group and the group with no addition (***p < 0.0001; by ANOVA).



Transporters Responsible for Organic Osmolyte Uptake by Post-Compaction Embryos

Although previous results clearly indicate that the GLYT1 transporter is the route of osmoregulated glycine uptake in cleavage stage embryos, the mechanisms mediating organic osmolyte accumulation in post-compaction stage embryos are uncertain. Using the four organic osmolytes (glutamine, glycine, L-alanine, β-alanine) that demonstrated osmoprotective capabilities in compaction-stage embryos, I attempted to determine the likely transport mechanisms responsible for uptake of each.

It has previously been demonstrated that glutamine, glycine and L-alanine are substrates of the broad-spectrum transporter B^{0+} that is present and highly active in the mouse blastocyst (Van Winkle et al., 1985) while β-alanine is a substrate of the β transport system that is present throughout preimplantation mouse embryo development (Van Winkle, 1994; Van Winkle 1996). Therefore it is hypothesized that the abovementioned transports are the likely routes of organic osmolyte accumulation in post-compaction stage embryos.

To test which transporters are used by the identified potential organic osmolytes, the rate of transport of each identified organic osmolyte in a tritiated form was measured in mouse embryos at the morula stage in the presence and absence of 10 mM of established competitive inhibitors for the amino acid transporter proposed to be responsible for transport. Morula were used on the assumption that transport at this stage will reflect the use of organic osmolytes during the transition from 8-cell to blastocyst. To test whether the B^{0+} transporter was responsible for accumulation of glutamine, glycine and L-alanine, morula were incubated for 10 min in the presence of 10 μM of the

^{3}H-labeled organic osmolyte with or without leucine, lysine or BCH (2-amino-endo-bycyclo[2.2.1]heptane-2-carboxylic acid) , established B^{0+} transport competitive inhibitors, each at 1000-fold excess over the ^{3}H-labeled test substrate (Van Winkle et al., 1990b). To test whether the β transporter is responsible for β-alanine accumulation, tritiated taurine was used as the test substrate, since a tritiated form of β-alanine was not available, and taurine and β-alanine share this transporter with similar affinities (Kwon and Handler, 1995; Chen et al., 2004). Mouse morula were incubated for 10 min in the presence of 10 μM of ^{3}H-taurine with or without 1000-fold excess β-alanine.

Each of the B^{0+} transport competitive inhibitors used here were able to nearly completely inhibit L-alanine accumulation, decreasing transport to nearly the background rate seen in 1000-fold excess unlabelled L-alanine (Fig. 27). Complete inhibition was observed in the presence of 1 mM of unlabeled L-alanine or 1 mM of leucine ($p < 0.001$, ANOVA). A similar result was observed with the addition of 1 mM of lysine or BCH ($p < 0.001$, ANOVA). Therefore the B^{0+} transporter appears to be responsible for the uptake of L-alanine in post-compaction stage embryos.

Excess unlabeled glycine completely inhibited ^{3}H-glycine uptake ($p < 0.001$, ANOVA), while the addition of leucine, lysine or BCH resulted in partial inhibition of ^{3}H-glycine accumulation (Fig. 28), with leucine inhibiting 60% of ^{3}H-glycine uptake ($p < 0.01$, ANOVA), lysine about 65% ($p < 0.01$, ANVOA) and BCH about 35%, although this did not reach significance ($p > 0.05$). This indicates that glycine uptake may be the result of more than one transporter at this stage of embryo development, since inhibition was not complete.

It was previously observed that some GLYT1 activity was present at the morula stage although the activity of GLYT1 was significantly decreased compared to earlier embryo stages. GLYT1 may therefore contribute to the portion of ^{3}H-glycine transport that was not inhibited. Therefore the rate of glycine uptake was measured in the presence of the GLYT1-specific inhibitor ORG23798 and 1000-fold excess of glutamine, a substrate of GLYT1. The addition of ORG23798 inhibited ^{3}H-glycine uptake to about 57% while glutamine had less of an effect, although neither reached significance (Fig. 28).

As the presence of B^{0+} transport competitive inhibitors results in the inhibition of approximately half of the ^{3}H-glycine accumulation and the presence of ORG23798 also appeared to decrease ^{3}H-glycine transport to approximately half (although the decrease was not significant), glycine transport in post-compaction stage embryos can be attributed to the B^{0+} transporter and possibly to some residual GLYT1 still present in morulae.

A significant decrease in ^{3}H-glutamine accumulation was observed in the presence of each of the established B^{0+} inhibitors (Fig. 29). Excess unlabeled glutamine inhibited approximately 90% of ^{3}H-glutamine uptake ($p < 0.001$, ANOVA). This indicates that the uptake of glutamine was attributed to specific saturable transport. A similar level of inhibition was seen with 1 mM of leucine ($p < 0.001$, ANOVA). In the presence of 1 mM of lysine or BCH only partial inhibition was observed. Lysine inhibited approximately 60% of ^{3}H-glutamine uptake ($p < 0.001$) and BCH 66% ($p < 0.001$). Like glycine, glutamine may also be transported by more than one transporter. Therefore the rate of glutamine uptake was measured in the presence of the GLYT1 specific inhibitor ORG23798 or glycine, as glutamine is a potential substrate of GLYT1.

While ORG23798 should only affect GLYT1, glycine is a substrate of both B^{0+} and GLYT1, and thus should compete for glutamine transport by either. The addition of ORG23798 inhibited approximately 30% of ^{3}H-glutamine uptake (Fig. 29), but this did not reach significance. Glycine, however, inhibited glutamine transport significantly, to about the same extent as lysine or BCH. Therefore, the transport of glutamine in post-compaction stage embryos can be attributed primarily to B^{0+}, while a small amount of glutamine may also be transported via residual GLYT1 activity still present in morulae, as indicated by a small, non-significant effect of ORG23798.

Unlabeled excess taurine was able to completely inhibit uptake of ^{3}H-taurine in morula (Fig. 30). The presence of 1 mM of unlabeled β-alanine resulted in a similar complete inhibition of ^{3}H-taurine accumulation. To demonstrate that B^{0+} was not responsible for accumulation of taurine, ^{3}H-taurine uptake was measured in the presence of the B^{0+} transport competitive inhibitor leucine. Leucine was not able to inhibit ^{3}H-taurine uptake. Therefore the β transport system is responsible for the transport of β-alanine in post-compaction stage embryos.

Figure 27: Rate of L-alanine uptake in the presence of B0+ transport inhibitors in mouse morula.

Rate of uptake of 1 μM [^{3}H]L-alanine in morula was determined in the presence and absence of 10 mM of B0$^+$ transport inhibitor (leucine, lysine or BCH) in 10 min. Each bar represents the mean ± SEM of 41-49 embryos in five replicates. Asterisks indicate a significant difference between a treatment group and the group with no addition. (***p < 0.0001; by ANOVA).

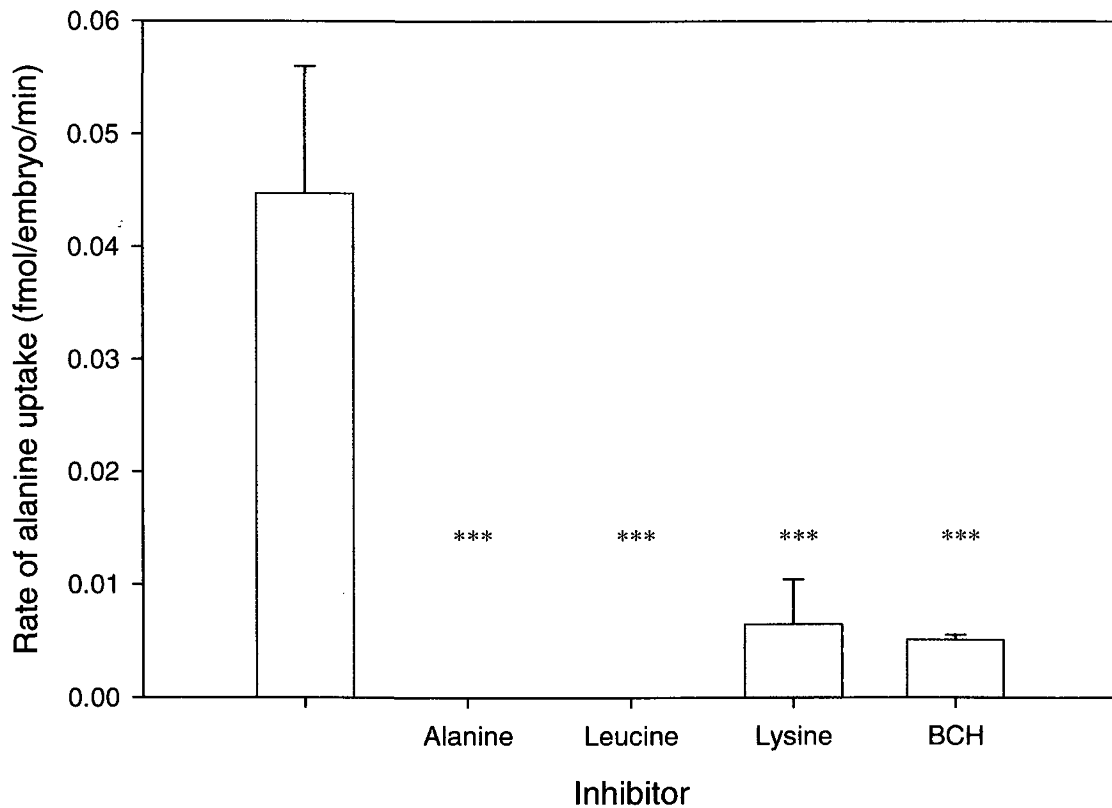


Figure 28: Rate of glycine uptake in the presence of B0$^+$ transport inhibitors and ORG23798 in mouse morula.

Rate of uptake of 1 μM [^{3}H]glycine in morula was determined in the presence and absence of 10 mM of B0$^+$ transport inhibitor (leucine, lysine or BCH) in 10 min. The rate of uptake of 1 μM [^{3}H]glycine in morula was also determine in the presence of 5μM ORG23798 or an equivalent amount of DMSO. Each bar represents the mean ± SEM of 49-50 embryos in five replicates. Asterisks indicate a significant difference between a treatment group and the group with no addition. (***$p < 0.0001$, **$p < 0.01$; by ANOVA).

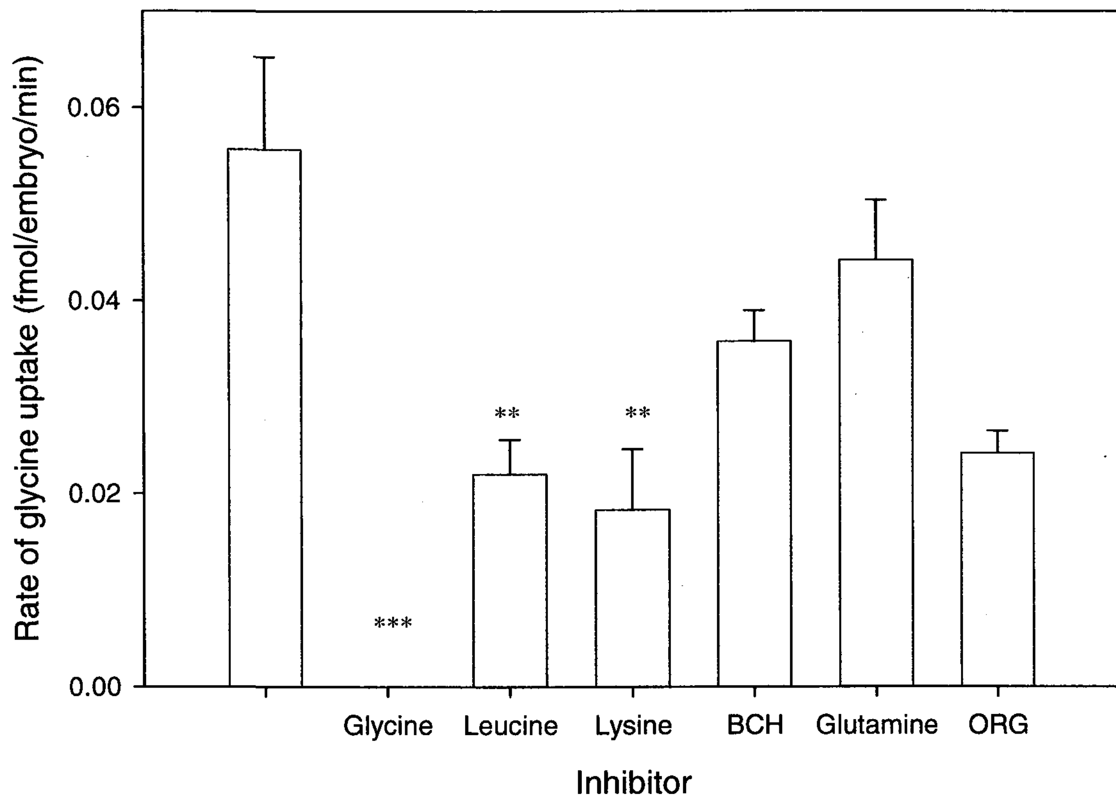


Figure 29: Rate of glutamine uptake in the presence of $B0^+$ transport inhibitors and ORG23798 in mouse morula.

Rate of uptake of 1 μM [^{3}H]glutamine in morula was determined in the presence and absence of 10 mM of $B0^+$ transport inhibitor (leucine, lysine or BCH) in 10 min. The rate of uptake of 1 μM [^{3}H]glutamine in morula was also determine in the presence of 5μM ORG23798 or an equivalent amount of DMSO. Each bar represents the mean ± SEM of 43-46 embryos in five replicates. Asterisks indicate a significant difference between a treatment group and the group with no addition. (***$p < 0.0001$; by ANOVA).

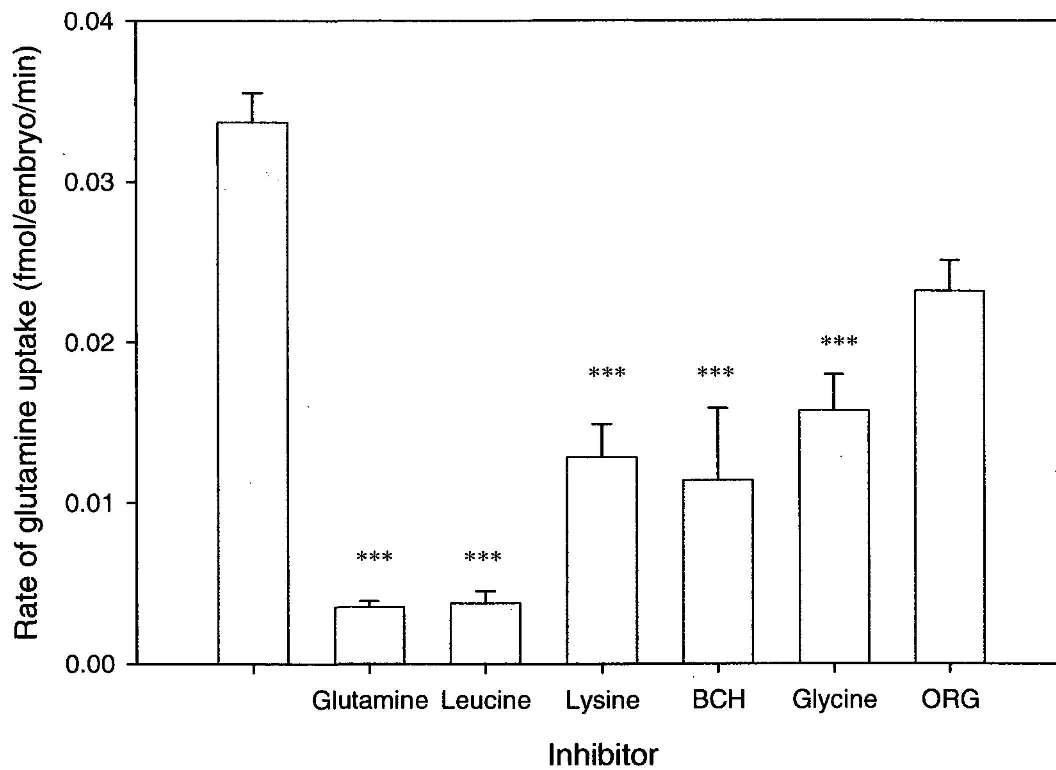
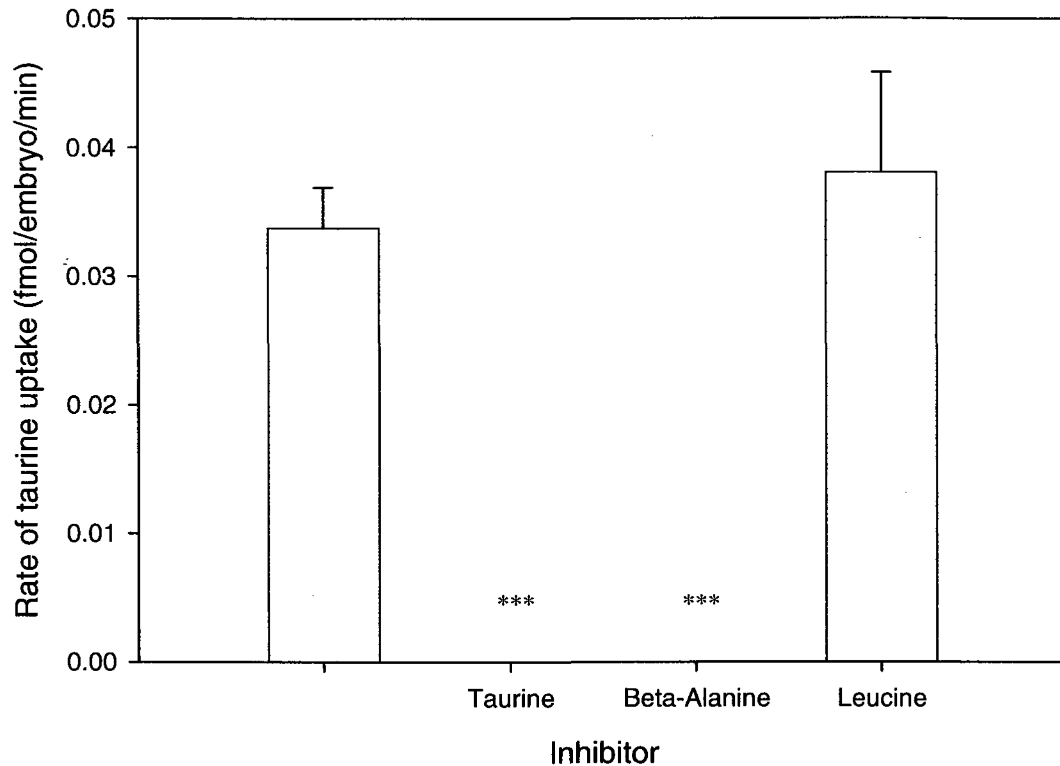


Figure 30: Rate of taurine uptake in the presence of system β transport inhibitor in mouse morula.

Rate of uptake of 1 μM [^{3}H]taurine in morula was determined in the presence and absence of 10 mM β-alanine in 10 min. Each bar represents the mean ± SEM of 44-47 embryos in five replicates. Asterisks indicate a significant difference between a treatment group and the group with no addition. (***$p < 0.0001$; by ANOVA).



DISCUSSION

GLYT1 Activity During Preimplantation Development

Glycine transport occurs at all stages of preimplantation mouse embryo development (Fig. 4). However we have shown here that the GLYT1 glycine transporter is not the major mechanism of transport of glycine into post-compaction embryos. It was observed that the GLYT1 glycine transporter became inactivated around the time of compaction after the 8-cell stage and before the blastocyst stage. The GLYT1-specific inhibitor ORG23798 completely inhibited the saturable uptake of glycine in embryos from the 1-cell to the 8-cell stages (Fig. 4). A small amount of GLYT1-independent saturable transport first appears at the morula stage, where glycine transport reaches a minimum. At this point, about half of saturable glycine transport is via residual GLYT1 activity, and half is resistant to ORG23798. A large amount of entirely GLYT1-independent (i.e., ORG23798-resistant) transport is present in blastocysts, as can be seen in Fig. 5. Therefore GLYT1 activity is only present in the cleavage stages of preimplantation mouse embryo development, largely absent by the morula stage, and undetectable in blastocysts.

These finding are consistent with previous reports which indicated that glycine transport that is effectively competed by only sarcosine, and hence was indentified as GLYT1 (designated "System Gly" in these earlier reports), is a significant component of glycine transport only until the 8-cell stage, but is not present in blastocysts (Van Winkle, 1988). The results reported here using ORG23798, which was previously shown to be a potent and specific inhibitor of GLYT1 (Steeves, 2003), conclusively show that the

earlier identification of a component of glycine transport that was high at the 1-cell and 2-cell stages, decreased before compaction, and absent in morulae is correct and also confirm the developmental changes in its activity.

The ability of glycine to be transported in post-compaction embryos in the presence of ORG23798 indicates that glycine is accumulated via a transporter other than GLYT1. In mouse blastocysts, the transporter $B^{0,+}$ has previously been shown to be highly active and responsible for virtually all glycine transport at that stage (Van Winkle, 1988). As GLYT1 activity is greatly decreased by the 8-cell stage, the large amount of glycine transport in blastocysts seen here that is not sensitive to ORG23798 was likely due to the $B^{0,+}$ activity that begins to develop in morulae and is highly active in blastocysts. This was confirmed by the results (Fig. 28) showing that the ORG23798-resistant component of glycine transport at the morula stage was inhibited by excess leucine and lysine, consistent with the characteristics of B^{0+} transport.

Effect of Increased Osmolarity on Compaction-Stage Embryo Development

Post-compaction stage embryos of CF1 mice are able to survive and develop at higher osmolarities in vitro than 1-cell zygote stage embryos. Mouse oviductal fluid is approximately 290-300 mOsM (Collins and Baltz, 1999). One-cell CF1 zygotes develop successfully beyond the 2-cell stage at osmolarities of 290 mOsM or less in vitro, while an osmolarity of 300 mOsM or higher is detrimental and induces a 2-cell block (Dawson and Baltz, 1997; Hadi et al., 2005). In contrast, the results reported here indicate that 8-cell embryos could develop to blastocysts in osmolarities up to 350 mOsM (although

blastocyst size and blastocoel cavity size were affected at the highest osmolarities) (Fig. 6).

While 1-cell, cleavage stage, and post-compaction stage mouse embryos are all sensitive to osmolarity, previous results and the results reported here indicate that the level of stress required to induce detrimental effects on development is different. An osmolarity of 300 mOsM or more has negative effects on mouse embryo development from the 1-cell zygote stage. In vitro development of in vivo-derived 2-cell embryos to blastocysts was supported in media with osmolarities up to 330 mOsM (Hadi et al., 2005). Here, it was found that the osmolarity must be raised above 360 mOsM to show the same negative effects on compaction-stage embryo development from the 8-cell stage to blastocysts (Fig. 6). Thus, 1-cell embryos are much more sensitive to increased osmolarity than later stages, and 2-cell embryos only somewhat more sensitive than 8-cell embryos.

It was also determined that 400 mOsM was the minimum osmolarity to reliably completely block development of 8-cell embryos to the blastocyst stage. Therefore the embryo culture osmolarity must be raised well above levels observed in vivo in order to effectively prevent post-compaction stage mouse embryo development. This indicates that while the earliest preimplantation embryos are very sensitive and may die due to relatively small increases in osmolarity; post-compaction stage embryos are quite robust and are able to survive in much more severe conditions.

Identification of Potential Organic Osmolytes Effective in Post-Compaction Embryos

A number of potential organic osmolytes were screened to determine if they could alleviate the detrimental effects of increased osmolarity at 400 mOsM on post-compaction stage embryo development. The most effective of these compounds were glutamine, glycine and L-alanine when added at 1 mM and β-alanine when added at 5 mM.

The osmoprotective abilities of β-alanine were similar in both cleavage-stage and post-compaction embryo stage development. The presence of β-alanine in embryo culture has previously been shown to effectively support development of zygotes to the blastocyst stage in raised osmolarity. The addition of β-alanine to embryo culture at a concentration of 5 mM conferred maximal development at higher osmolarities as compared to the lower development it supported when added to culture at a concentration of 1 mM (Dawson et al., 1997). This is similar to what was observed in post-compaction stage embryos. The addition of β-alanine at 1 mM was not effective in sustaining post-compaction stage mouse embryo development at 400 mOsM; while the presence of β-alanine at 5 mM promoted successful development of post-compaction stage mouse embryos to blastocysts. β-alanine is transported via system β in cleavage-stage embryos (Dawson et al., 1997). The system β transport mechanism is present at every stage of preimplantation mouse embryo development (Van Winkle, 1994; Van Winkle 1996). Therefore β-alanine can be transported via the system β transport mechanism to be utilized to support maximal development of post-compaction stage mouse embryos in raised osmolarity.

Van Winkle et. al (1990a) have shown that L-alanine protects 2-cell mouse embryo development to the blastocyst stage against increased osmolarity. Like cleavage stage-embryos, L-alanine provided osmoprotection to post-compaction stage embryos in the presence of raised osmolarity. L-Alanine is not transported to any appreciable extent before the 8-cell stage but transport increases substantially at the blastocyst stage by the B^{0+} transport system (Van Winkle et al. 1990b). Thus L-alanine is effectively transported in post-compaction stage embryos and can be utilized to support optimal development.

Both glutamine and glycine provide osmoprotection to cleavage-stage embryo development (Dawson and Baltz, 1997). As in cleavage-stage embryos, glutamine and glycine protect post-compaction stage embryos against raised osmolarity. The Gly transporter GLYT1 in cleavage-stage embryos is responsible for the transport of its high affinity substrate glycine, it also accepts glutamine with low affinity (Steeves, 2003). However the activity of GLYT1 is greatly decreased by the 8-cell stage and is not present in blastocysts, as shown here (above) and previously reported (Van Winkle, 1988). At the blastocyst stage, a broad-spectrum transporter, system B^{0+}, is actively transporting amino acids including glutamine, glycine and L-alanine (Van Winkle, 1990b). Therefore glutamine and glycine can be effectively transported into post-compaction stage embryos to provide protection against increased osmolarity.

Betaine, proline, taurine and myo-inositol did not protect embryos against the detrimental effects of increased osmolarity. Betaine and proline have both been shown to act as organic osmolytes in cleavage-stage embryos (Biggers et. al. 1993; Hammer and Baltz, 2002; Dawson and Baltz, 1997), however they were ineffective in post-compaction stage embryo development. This is likely due to the fact that the betaine and proline

transporter, SIT1, in preimplantation embryos is active only at the 1-cell and 2-cell stages, and is completely inactive by the 8-cell stage (Anas et al., 2008). As in post-compaction stage embryos, myo-inositol and taurine do not act as organic osmolytes in cleavage-stage embryos (Dawson and Baltz, 1997; Hammer and Baltz, 2003); although myo-inositol transport can be demonstrated in preimplantation mouse embryos (Kane et al., 1992) and taurine is transported via system β, which is present at each stage of preimplantation development (Van Winkle, 1994; Van Winkle 1996).

Rate of Organic Osmolyte Accumulation as a Function of External Osmolarity

The main test that determines whether an organic osmolyte is likely used by a given cell type is its ability to protect viability when osmolarity is increased. Four compounds were identified here that have this property: glutamine, glycine, L-alanine and β-alanine. However, organic osmolytes in other cell types, and in 1-cell embryos, also exhibit a second key property, which is that their intracellular accumulation is dependant on external osmolarity (Baltz, 2001). Here, three of the organic osmolytes found to be effective in post-compaction stage embryos were tested to determine whether organic osmolyte accumulation increases with increasing osmolarity. The total amount of glutamine, glycine and L-alanine accumulated by post-compaction embryos was, however, not significantly affected by external osmolarity. Thus the accumulation of organic osmolytes is not regulated by external tonicity in post-compaction stage embryos, in contrast to 1-cell embryos.

These findings are in contrast with the classical behavior of an organic osmolytes as seen, for example, in the kidney. In the kidney, cellular osmotic balance is maintained

through the accumulation of compatible organic osmolytes via osmolyte transporters such as the betaine/GABA transporter BGT1. Up-regulation of transport is stimulated by hypertonicity. Similarly, Garcia-Perez and Burg (1991) demonstrated that increased external osmolarity causes an increase in intracellular betaine accumulation in renal cells. A similar result was observed in cleavage-stage embryos for glycine and betaine. Intracellular glycine accumulation nearly doubles when osmolarity is raised from 250 mOsM to 310 mOsM (Dawson et al., 1998).

Although glutamine, glycine and L-alanine do not accumulate at higher levels in increased osmolarity, they may still function as non-standard organic osmolytes. First, it has previously been stated that post compaction-stage mouse embryos are able to survive at higher osmolarities in vitro than cleavage-stage embryos. Therefore post compaction-stage embryos may not need to accumulate organic osmolytes at the same levels as cleavage-stage embryos in order to be provided with the similar level of osmoprotection. Also in compaction-stage embryos, glutamine, glycine and L-alanine all behave in a similar manner to alleviate the detrimental effects of high osmolarity, consistent with that of an organic osmolyte. It is not likely that they are being utilized for a different role in post-compaction stage embryos as they react in the same way despite the fact that they are not related biochemically and metabolically. Thus, for example, they are unlikely to be used in the same metabolic pathway.

How, then, do these compounds protect development of 8-cell embryos to blastocysts when they are not accumulated to a greater level when external osmolarity is increased? We hypothesize that the level of accumulation is sufficient to displace a significant amount of intracellular ions, lowering the baseline amount of total ions in the

embryos and therefore decreasing intracellular ionic strength. Even though the level of presumed organic osmolytes apparently does not increase as the external osmolarity is raised, it nonetheless must lower the baseline intracellular ionic strength enough that the additional ions presumably accumulated by the embryos at higher osmolarities still does not rise above the threshold that would prevent development. Thus, it is proposed that, even in the absence of active regulation of accumulation of L-alanine, glycine or glutamine, these compounds are accumulated and displace enough intracellular ions that development can still occur in vitro even well above physiological osmolarity.

Effect of Organic Osmolytes on Blastocyst Volume

Organic osmolytes have previously been reported to confer on 1-cell embryos the ability to maintain their volumes in the presence of high osmolarity (Steeves et al, 2003). We tested whether organic osmolytes had a similar effect on the size of post-compaction stage embryos. Blastocysts were measured after embryos were cultured for 48 hours from the 8-cell stage in KSOM at 250, 310, 340, 370 or 400 mOsM, to see if there was a difference in blastocyst volume at the various osmolarities in the presence or absence of organic osmolytes. The size of the embryos on day 3 and day 4 of culture were larger in media from 310 to 400 mOsM in the presence of each of the four organic osmolytes, glutamine, glycine, L-alanine and β-alanine (Fig. 24).

As previously shown post-compaction stage embryos can successfully develop to blastocysts at an osmolarity of 310 mOsM without the addition of any organic osmolyte. Therefore, organic osmolytes are not absolutely needed for the development to blastocysts of post-compaction stage embryos cultured at an osmolarity found in vivo.

However the addition of organic osmolytes at 310 mOsM resulted in a significant increase in blastocyst volume, indicating that there is a similar benefit to having any of these compounds present during culture from the 8-cell stage in vitro. It cannot be ruled out that the effect on blastocyst volume is due to an effect of each compound that is independent of any role as an organic osmolyte. However, as discussed above, it would seem unlikely that biochemically unrelated compounds, each of which protect development against increased osmolarity, would have another, unrelated protective effect in common. Therefore, these compounds appear to be beneficial for optimal growth of post-compaction mouse embryo development by functioning as organic osmolytes even at the physiological osmolarity found in vivo.

Effect of the Identified Organic Osmolytes on Cell Number in Blastocysts

Since organic osmolytes are capable of protecting post-compaction stage embryo volume in the presence of increased osmolarity, we wanted to see if organic osmolytes would also have an effect on total cell number in the face of high osmolarity. Mouse blastocyst were stained with a DNA-binding dye after being cultured for 48 hours from the 8-cell stage in 310 or 370 mOsM KSOM with each of the four organic osmolytes (glutamine, glycine, L-alanine, or β-alanine) or without.

At 310 mOsM there was no difference in cell number with organic osmolyte than without. Consequently total cell number of blastocyst culture from 8-cells was not affected by an increased osmolarity of 310 mOsM.

As stated earlier, 310 mOsM is close to the physiological osmolarity of mouse oviductal fluid (Collins and Baltz, 1999) and post-compaction stage embryos can survive

successfully at this osmolarity. As a result, post-compaction stage embryos may not be under significant osmotic stress as to see a depression in cell number at 310 mOsM. Thus, at physiological osmolarity, the presence of organic osmolytes appears to support a greater expansion of blastocysts, resulting in a greater total volume (above), but this greater size is not accompanied by an increase in cell number. Thus, it can be inferred that the presence of organic osmolytes at physiological osmolarity results in increased ability of the developing blastocyst to transport fluid across the trophectoderm, but does not affect cell proliferation.

At 370 mOsM, however, there was a significant difference in cell number between blastocyst cultured with organic osmolyte than without. At 370 mOsM in the absence of any organic osmolytes, there was a decrease in the total cell number in mouse blastocysts. The addition of organic osmolytes at 370 mOsM resulted in total blastocyst cell number similar to blastocysts cultured at 310 mOsM. Therefore, when osmolarity is increased well above the physiological range, cell proliferation is decreased and blastocysts develop with fewer total cells. The addition of organic osmolytes at these high osmolarities rescues cell proliferation. Therefore, there appears to be two thresholds for effects of osmolarity and organic osmolytes on blastocyst development. At near-physiological levels, blastocyst development is not affected by the presence or absence of organic osmolytes, but the ability of the blastocyst to expand is increased by the presence of organic osmolytes. At much higher levels of osmotic stress, development of blastocysts is impaired and there is less cell proliferation and fewer cells in the blastocysts, and this is rescued by the addition of organic osmolytes.

Transporters Responsible for Organic Osmolyte Uptake by Post-Compaction Embryos

The mechanism of organic osmolyte transport in post-compaction stage embryos was unknown. The results obtained here indicate that the transport of the four organic osmolytes effective in post-compaction stage embryos, glutamine, glycine, L-alanine, and β-alanine, may be accounted for by the activity of two transport systems: B^{0+} and the β transporter. B^{0+} is a sodium dependant transporter of basic, zwitterionic and bicyclic amino acids and it is present and highly active in mouse blastocysts, which is highly susceptible to competitive inhibition by leucine, lysine and BCH (Van Winkle et al., 1985). As shown in the Results section, transport of L-alanine was entirely inhibited by competitive inhibitors of B^{0+}, while substantial portions of both glycine and glutamine transport were blocked by these inhibitors. Thus, transport of L-alanine, glycine and glutamine are likely mediated all or in part by B^{0+}. The remaining portion of glycine and glutamine transport in morulae may be by GLYT1, since there was inhibition by the GLYT1 inhibitor ORG23798, and since transport measurements in morulae showed a very small component of glycine transport that is via GLYT1. However, this transport should not contribute significantly to total accumulation of glycine or glutamine during development to blastocysts, since GLYT1 entirely disappears and B^{0+} becomes highly active. Therefore, it is likely that the beneficial effect of L-alanine, glycine and glutamine on development from the 8-cell to blastocyst stages is due to their transport via B^{0+}.

The last of the four organic osmolytes that was effective in post-compaction stage embryos, β-alanine, is a known substrate of the system β transporter (Dawson and Baltz,

1997). The system β transporter is present throughout preimplantation development and no other transport systems have been found to carry β-amino acids in preimplantation embryos (Van Winkle, 1994; Van Winkle 1996). Here, any transport of taurine by B^{0+} was ruled out by showing that it was not inhibited by leucine. Therefore, the effects of β-alanine are almost certainly mediated by system β transport.

In summary, the uptake and accumulation of the compounds identified here to be protective against increased osmolarity are likely mediated by two transporters (Table II). The first is B^{0+}, which accounts for most or all of L-alanine, glycine and glutamine transport and accumulation from the 8-cell to blastocyst stage. This transporter appears at the morula stage and only becomes highly active in blastocysts. Thus, the accumulation of L-alanine, glycine and glutamine are due to a transport property specific to blastocysts. In contrast, system β is present throughout preimplantation development, and thus β-alanine may be accumulated and retained throughout preimplantation development and is not specific to the blastocyst.

Table II: Potential organic osmolyte transporters in post-compaction stage embryos.

Organic Osmolyte	Transporter in 1-cell and Cleavage-Stage Embryos	Transporter in Post-Compaction Stage Embryos
Glutamine	GLYT1	B^{0+}, small amount via GLYT1 at morula stage
Glycine	GLYT1	B^{0+}, small amount via GLYT1 at morula stage
β-alanine	β transporter	β transporter
L-alanine	very little transport in early embryos	B^{0+}

SIGNIFICANCE

It has been observed that cleavage-stage mouse embryos of most genotype backgrounds cannot develop in vitro when medium osmolarity is raised to the levels found in mouse oviductal fluid; approximately 300 mOsM in the absence of organic osmolytes. The addition of organic osmolytes such as glycine, glutamine, betaine and proline to culture media was able to alleviate the detrimental effects of high osmolarity on development of mouse embryos from the 1-cell stage in vitro (Dawson and Baltz, 1997). In contrast, post-compaction mouse embryos are able to successfully develop in culture when medium osmolarity is raised to physiological levels found in vivo without the addition of organic osmolytes. Therefore organic osmolytes are not needed for in vitro post-compaction embryo development. However the addition of organic osmolytes in culture at osmolarities found in vivo resulted in an increase in blastocyst volume. This work establishes that, although organic osmolytes are not absolutely required for post-compaction embryo development to blastocysts at increased osmolarities, they are needed for optimal expansion of post-compaction stage embryos at physiological osmolarity, and to maintain cell proliferation at high osmolarities under conditions of osmotic stress.

Therefore organic osmolytes can play an important role in providing protection to post-compaction stage embryos during cryopreservation. During cryopreservation embryos are subject to osmotic stress as rise in osmolarity occurs during freezing due to a change in external and to a smaller extent internal concentrations of salts changes (Ashwood-Smith, 1986). The increase in osmolarity causes the embryo to shrink as water leaves the cells (Ashwood-Smith, 1986). As previously mentioned organic osmolytes can

help to increase post-compaction embryo volume in presence of raised osmolarity and may be able to do so during cryopreservation.

In addition, post-compaction stage mouse embryos are more robust than cleavage-stage mouse embryos. Therefore slight deviations in osmolarity or availability of organic osmolytes, in vivo or in embryo culture in vitro, may be detrimental to cleavage-stage embryo development and result in embryos death, while post-compaction stage embryos are more likely to survive and develop under the same stress.

Therefore post-compaction stage embryos are more successful for in vitro clinical embryo culture. Post-compaction stage embryos would be able to survive small perturbations in the culture environment that may affect the media osmolarity.

CONCLUSION

GLYT1 is responsible for glycine accumulation in cleavage-stage embryos and is no longer active after the morula stage. Glycine transport in post-compaction stage embryos occurs via a different transporter than GLYT1. Therefore, the major cell volume-regulatory mechanism identified in the 1-cell embryo is no longer present after the cleavage stages and cannot be involved in cell volume regulation or osmoprotection in embryos after compaction.

Post-compaction stage embryos are viable at high osmolarities that have shown to be detrimental to the development of 1-cell embryos. The osmolarity must be raised to 400 mOsM, well above the osmolarity observed in vivo, in order to consistently completely block 8-cell embryo development to the blastocyst stage. Therefore, there is no absolute block to development from the 8-cell stage at any osmolarity near those found in vivo, in contrast to development from the 1-cell stage.

Four organic osmolytes that provide osmoprotection to cleavage-stage mouse embryos, glutamine, glycine, L-alanine and β-alanine act as organic osmolytes in post-compaction stage mouse embryos. These however do not act like standard organic osmolytes in somatic cells or 1-cell embryos since their accumulation is not increased with increased osmolarity.

These organic osmolytes are able to maintain blastocyst volume at physiological osmolarity. Therefore, they may have a necessary protective role in vivo for optimal blastocyst development.

When osmolarity was raised substantially above physiological levels, blastocyst cell number was significantly decreased, while any of the four organic osmolytes rescued

cell proliferation. Therefore, these organic osmolytes are protective against the effects on embryo cell development of osmotic stress in vitro.

Post-compaction stage embryos have at least two transporters that allow the embryo to accumulate compounds that act as organic osmolytes, the system β transporter and the B^{0+} transporter. The β transporter is also present throughout preimplantation development, but B^{0+} activity appears after compaction. Thus, the array of transporters of compounds that act as organic osmolytes in preimplantation embryos changes at compaction.

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