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Mouse embryo development following IVF in media containing either L-glutamine or glycyl-L-glutamine

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BACKGROUND: The development of the mouse zygote following fertilization *in vitro* in a KSOM-type medium containing either L-glutamine or glycyl-L-glutamine has been examined, and compared with the development of mouse zygotes produced by natural fertilization. METHODS: Mouse IVF, embryo culture and embryo transfer. RESULTS: Fertilization rates, development to the blastocyst stage, implantation rate, gross fetal development and fetal body weight are not different in a KSOM-type medium containing either L-glutamine or glycyl-L-glutamine. No evidence of abnormal fetal development, such as exencephaly, was observed. The replacement of L-glutamine with glycyl-L-glutamine favoured the development of relatively more inner cell mass cells than trophectoderm cells, and reduced the numbers of pyknotic and fragmented nuclei in the blastocysts that developed *in vitro*. CONCLUSIONS: There is no evidence that the presence of glutamine in the medium used for IVF influences significantly the subsequent development of the zygote. Replacing glutamine with glycyl-L-glutamine may be advantageous.

Key words: exencephaly/glutamine/glycylglutamine/IVF

Introduction

There has been renewed interest on the need for and role of amino acids (AA) in chemically defined media for the culture of preimplantation mammalian embryos (reviewed in Gardner, 1994; Gardner and Lane, 1999; Biggers, 1998, 2002, 2003; Summers and Biggers, 2003). L-glutamine (Gln), which is included in the majority of chemically-defined media that have been designed for the culture of preimplantation mammalian embryos (for review, see Summers and Biggers, 2003), has received particular scrutiny. A potential drawback to the use of Gln, however, is its inherent chemical instability, causing it to undergo spontaneous breakdown into equimolar amounts of ammonium and 5-pyrrolidone-2-carboxylic acid (for review, see Greenstein and Winitz, 1961), the products of which may be deleterious to the developing embryo. Inhibitory effects of Gln on preimplantation embryo development were shown by Devreker and Hardy (1997) using zygotes of an F1 hybrid strain cultured in KSOM and Gln-free KSOM. They observed no difference in the proportion of zygotes developing to the blastocyst stage in the two media, but blastocysts derived from culture in Gln-free KSOM had higher cell counts. These results have recently been confirmed using AA-supplemented KSOM (Biggers *et al.*, 2004a).

Serious concern arose in the human IVF community about putative teratogenic effects of ammonium accumulation when Lane and Gardner (1994) reported cases of exencephaly in mice following the transfer into surrogate mothers of blastocysts cultured in medium mMTF containing Gln. This concern was further enhanced by the observation that cases of exencephaly also arose in fetuses that developed from blastocysts produced in the presence of ammonium chloride. The toxic effect of Gln can be avoided by making use of the fact that dipeptides can be directly used by cells *in vitro* (Eagle, 1955). Thus, Lane *et al.* (2001) replaced Gln with L-alanyl-L-glutamine (AlaGln) for the culture of mouse preimplantation embryos, a practice that has been adopted by several commercial companies that sell media for the culture of human preimplantation embryos (e.g. G-1™ version 3 medium, Vitrolife, Göteborg, Sweden; Global®, LifeGlobal, Canada; Quinn's Advantage®, Cooper Surgical, Trumbull, CT). Recently, we have reported that glycyl-L-glutamine (GlyGln) seems a preferable choice of a dipeptide since it differentially favors the development of the inner cell mass (ICM) (Biggers *et al.*, 2004a).

The teratological effects of ammonium accumulation described by Lane and Gardner (1994) have not been confirmed by others (Sinawat *et al.*, 2003; Biggers *et al.*, 2004a). The reasons for the differences are unknown but may be related to differences in the genetic makeup of the strains of mice used (for review, see Biggers *et al.*, 2004b). The work done so far has only reported the effects of Gln on zygotes

produced *in vivo*. No comparative studies have been reported in which unfertilized ova were exposed either to Gln or GlyGln before and during the fertilization process. In this paper we describe the results of an experiment in which fertilization occurred *in vitro* in a medium containing Gln at twice the concentration used routinely in embryo culture media. Parallel studies were perormed using a similar concentration of GlyGln.

Materials and methods

Animals

Embryos were produced by mating outbred CF1 females (6–8 weeks old; Harlan-Sprague-Dawley) with hybrid BDF males (2–11 months old; B6D2F1/CrlBR; Charles River Laboratories). Outbred CD1 females (Taconic, Germantown, NY) mated to vasectomized outbred CD1 males provided pseudopregnant females to serve as surrogate mothers for embryo transfers. Animals were killed by cervical dislocation. The animals used in this study were maintained in accordance with the guidelines of the Committee of Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council (DHSS publication No. NIH 85–23, revised 1985).

Culture media

All culture media were formulated from KSOM, which contains 1.0 mg/ml BSA (Fraction V; Sigma, Cat. # A9647; lot # 15H0672) (Lawitts and Biggers, 1993). Medium mKSOM is KSOM where the concentration of BSA is raised to 4.0 mg/ml and supplemented with 5.56 mmol/l glucose (Summers *et al.*, 1995, 2000). KSOM containing 5.56 mmol/l glucose is designated as $KSOM_g$. KSOM, $KSOM_g$ and mKSOM containing AA are designated as $KSOM^{AA}$ $KSOM_g^{AA}$ and $mKSOM^{AA}$, respectively (Table I). $KSOM_g^{AA}$ when supplemented with GlyGln at 2 mmol/l is designated as GlyGln-$KSOM_g^{AA}$. $KSOM_g^{AA}$ was also supplemented with Gln at 2 mmol/l and is designated as Gln-$KSOM_g^{AA}$; this is in contrast with the concentration of 1 mmol/l Gln normally used in KSOM (Lawitts and Biggers, 1993). The concentrations of Gln and GlyGln were increased to be consistent with the concentration of GlyGln to exaggerate any effect of ammonium that might result from the chemical breakdown of Gln.

KSOM and mKSOM was prepared from frozen stocks, which were thawed, prepared and supplemented as required for each culture treatment (Biggers *et al.*, 1997).The AA stock solutions were stored at 4 °C. The non-essential AA (NEAA) and the essential AA (EAA) in $KSOM_g^{AA}$ (Table I) were added in concentrations of one half of the amounts present in Modified Eagle's Medium (Eagle, 1959). All chemicals were from Sigma, except Eagle's NEAA (#11140-019) and Eagle's EAA (#11130-010) (Gibco BRL, Life Technologies, Inc., Grand Island, NY).

Sperm, oocyte and zygote collection

Gametes were collected using the methods described elsewhere (Summers *et al.*, 1995, 2000).

Fertilization in vitro

Fertilization *in vitro* was done in 1 ml drops of $mKSOM^{AA}$ supplemented with either 2 mmol/l Gln or 2 mmol/l GlyGln under mineral oil as reported in detail previously (Summers *et al.*, 1995, 2000). The concentration of sperm was $\sim 10^6$/ml.

Table I. Composition of medium designated as $KSOM_g^{AA}$

Component	Concentration (mmol/l)
NaCl	95
KCl	2.5
KH_2PO_4	0.35
$MgSO_4$	0.2
Lactate	10
Pyruvate	0.2
Glucose	5.56
$NaHCO_3$	25
$CaCl_2$	1.71
L-glutamine[a]	1.0
EDTA	0.01
BSA[b]	1.0 mg/ml
L-alanine–HCl	0.05
L-arginine–HCl	0.3
L-asparagine–H_2O	0.05
L-aspartic acid	0.05
L-cystine	0.05
L-glutamic acid	0.05
Glycine	0.05
L-histidine–HCl–H_2O	0.1
L-isoleucine	0.2
L-leucine	0.2
L-lysine–HCl	0.2
L-methionine	0.05
L-phenylalanine	0.1
L-proline	0.05
L-serine	0.05
L-threonine	0.2
L-tryptophan	0.025
L-tyrosine	0.1
L-valine	0.2

Concentrations of amino acids listed above are used only at half-strength Eagles (1959).
[a]L-glutamine in the present study was used at a concentration of 2 mmol/l; the medium is designated Gln-$KSOM_g^{AA}$.
[b]$KSOM_g^{AA}$ contains 1.0 mg/ml BSA. Modified $KSOM^{AA}$, designated $mKSOM^{AA}$ contains 4.0 mg/ml BSA and is the medium used for fertilization *in vitro*.

Embryo culture

Ova that had been exposed to sperm from fertilization *in vitro* and *in vivo* were cultured in groups of 12 in 50 μl droplets of medium consisting of either Gln-$KSOM_g^{AA}$ or GlyGln-$KSOM_g^{AA}$ overlaid with mineral oil. The ova were allotted at random to the two media. The embryos were incubated at 37 °C in modular incubator chambers (Billups-Rothenberg Inc., Del Mar, CA) which were gassed with a mixture of 5% O_2, 6% CO_2 and 89% N_2 (Lawitts and Biggers, 1993). Culture plates (60 mm suspension, non-tissue cultured-treated; Corning Inc., Corning, NY) were prepared 1 day before embryo collection and equilibrated in the module overnight.

Embryo evaluation

Embryos were observed at ×40 to ×100 on a warmed (35 °C) microscope stage of a Wild dissecting microscope. The numbers of zygotes that cleaved and developed by 96, 120 and 144 h post-hCG administration into zona-enclosed blastocysts, and partially and completely hatched blastocysts, were counted.

Embryo transfer

Some of the cultured morulae and blastocysts derived 96 h post-hCG in either Gly-$KSOM_g^{AA}$ or GlyGln-$KSOM_g^{AA}$ were transferred into day 3 pseudopregnant females; six embryos chosen at random to each uterine horn. The embryos produced in each medium were allotted at random to either the left or right uterine horn. The operator performing the embryo transfer experiments was blinded as to the embryo source (i.e. zygotes derived from fertilization *in vitro* or mated females) and culture conditions used. The embryos were

allowed to develop for a further 11–12 days. Thus, the fetuses that developed were 14–15 days post-hCG. At this time the females were killed and the numbers of implantation sites and fetuses counted in each horn. All the fetuses were assessed for gross abnormalities and weighed.

Differential ICM and TE cell counts

The remaining cultured blastocysts obtained 144 h post-hCG were fixed and differentially stained to determine the cell count of the inner cell mass (ICM) and trophectoderm (TE). The method is based on modifications of a method originally described using polynucleotide-specific fluorochromes (Handyside and Hunter, 1984, 1986; Papaioannou and Ebert, 1988). The complete method of differential staining has been described elsewhere (Biggers *et al.*, 2000). The numbers of non-mitotic, mitotic, pyknotic and fragmented nuclei were also counted.

Experimental design and biometrical considerations

Zygotes were produced by IVF in either Gln-mKSOM^{AA} or GlyGln-mKSOM^{AA}, and then transferred in groups of 12 to Gln-KSOM$_g^{AA}$ and GlyGln-KSOM$_g^{AA}$ for the rest of the culture period, respectively. Groups of 12 zygotes, produced by fertilization *in vivo* (controls), were also cultured simultaneously in Gln-KSOM$_g^{AA}$ and GlyGln-KSOM$_g^{AA}$. The experimental unit was thus a droplet containing 12 zygotes. The four treatments were compared in a randomized block experiment with unequal numbers of experimental units in each cell. Four replicates were done. The number of intact 1-cell embryos that were exposed to sperm 24 h post-hCG were assumed to be fertilized. The 2-cell embryos were cultured for a further 72 h (96 h post-hCG) at which time the numbers of blastocysts that had developed were counted. The drops containing embryos were then divided at random into two groups. In the first group the morulae and blastocysts that had developed were transferred into the uteri of surrogate mothers. The second group was cultured for a further 48 h (144 h post-hCG) and at 120 and 144 h post-hCG the numbers of zona-enclosed blastocysts, partially hatched and completely hatched blastocysts, were counted. The blastocysts that developed by 144 h post-hCG were then stained for counting the numbers of ICM and TE cells and pyknotic and fragmented nuclei.

Two sets of observations were made. Set 1 were the categorical scores that recorded the morphological development of the zygotes into 2-cell stages, blastocysts by 96, 120 and 144 h post-hCG, including the numbers of zona-enclosed blastocysts, hatching and completely hatched blastocysts. Before applying exploratory graphical and statistical analyses, the data were re-expressed as the numbers of embryos that at least developed into blastocysts, at least started to hatch and at least completely hatched. The data was then analyzed using the parametric generalized linear model (McCullagh and Nelder, 1989), assuming that the errors follow a binomial distribution. The second set of observations were the counts of ICM and TE cells in each blastocyst that had developed 144 h post-hCG in each culture condition. The marginal distributions are displayed using either barplots or notched box plots. The boxplots show the 10th, 25th, 50th (median), 75th and 90th percentiles; observations outside of the 10th and 90th percentiles are considered outliers. The notches on the boxplots are the 95% median confidence limits. Two medians are significantly different if their confidence limits do not overlap. In the cell counts the errors are assumed to be formed by a multiplicative process. Thus the data have been submitted to an analysis of variance (ANOVA) assuming lognormally distributed errors. The numbers of dead cells are assumed to follow a Poisson distribution and have also been analyzed by ANOVA after transformation to the logarithmic scale.

The embryo transfer data (embryos that at least implanted and the numbers of fetuses) were treated as two sets of stratified 2 × 2 contingency tables (Mehta and Patel, 2001), each stratum being one mother. The exact probability that the sets were homogeneous was calculated. If $P > 0.05$, the pooled exact estimate of the common odds ratio and its confidence limits ($P = 0.95$) was then computed. The effects of the two media were considered significantly different if the confidence limits did not include 1.

All but one of the statistical analyses were done using the S-Plus 5 package (Insightful, Seattle, WA). The exact analysis of stratified 2 × 2 contingency tables was computed using StatXact 5 (Cytel Software Corporation, Cambridge, MA). A difference in all analyses is considered statistically significant if $P < 0.05$.

Results

Development to the 2-cell stage

The percentages of fertilized ova that cleaved to the 2-cell stage after 24 h in Gln-KSOM$_g^{AA}$ and GlyGln-KSOM$_g^{AA}$ are summarized in Table II. A high rate of fertilization and cleavage to the 2-cell stage was obtained in both media: Gln-KSOM^{AA} (95–97) and GlyGln-KSOM^{AA} (95–96). There was no difference in the rates of fertilization and cleavage to the 2-cell stage between the different treatment groups ($P = 0.653$).

Development to the blastocyst stage

Tables II and IIIA summarize the percentages of 2-cell embryos derived from either fertilization *in vitro* or *in vivo* that developed to the blastocyst stage by 96, 120 and 144 h post hCG administration. Table IIIB records the percentage of blastocysts that either partially or completely hatched by 120 and 144 h post-hCG administration. A high percentage of both *in vitro*- and *in vivo*-derived zygotes developed at least to the blastocyst stage in both Gln-KSOM$_g^{AA}$ and GlyGln-KSOM$_g^{AA}$. The rate of blastocyst development by 96 h and 120 h post-hCG administration was slower in the group of zygotes derived from fertilization *in vitro* compared to *in vivo*. This effect, however, is less pronounced in embryos cultured in GlyGln-KSOM^{AA} when compared to Gln-KSOM^{AA}. There was no difference in the percentage of 2-cell embryos that developed at least to the zona-enclosed blastocyst stage at 144 h post-hCG administration in the four treatment groups ($P = 0.308$). The percentage of blastocysts that partially or completely hatched, however, was dependent on both the method of fertilization and on the culture medium. A lower percentage of blastocysts derived from fertilization of ova

Table II. The effects of method of fertilization in either Gln-KSOM$_g^{AA}$ or GlyGln-KSOM$_g^{AA}$ on the incidence of fertilization and the incidence of blastocyst formation 96 h post-hCG

Fertilization site	Peptide	Ova exposed to sperm (*n*)	2-cell (*n*)	Blastocysts (*n*)
In vitro	Gln	245	237 (96.7%)	2 (0.8%)[a]
In vitro	GlyGln	246	237 (96.3%)	0
In vivo	Gln	190	181 (95.3%)	6 (3.3%)
In vivo	GlyGln	202	191 (94.6%)	10 (5.2%)
Fisher's exact test (*P*)			0.653	0.0002

[a]Percentage of blastocysts developing from 2-cells.

Table III. The effects of method of fertilization in either Gln-KSOM$_g^{AA}$ or GlyGln-KSOM$_g^{AA}$ on the incidence of blastocyst formation and hatching 120 and 144 h post-hCG (the 2-cell embryos are those not used for embryo transfer)

A. Development of blastocysts

Fertilization site	Peptide	Ova exposed to sperm (*n*)	2 cells (*n*)[a]	Blastocysts (120 h post-hCG) (*n*)	Blastocysts (144 h post-hCG) (*n*)
In vitro	Gln	142	137 (96.5%)	56 (40.9%)[b]	113 (82.5%)[b]
In vitro	GlyGln	133	128 (96.2%)	71 (55.5%)	99 (77.3%)
In vivo	Gln	83	77 (92.8%)	57 (74.0%)	67 (87.0%)
In vivo	GlyGln	95	87 (91.6%)	65 (74.7%)	74 (85.0%)
Fisher's exact test (*P*)			0.258	0	0.308

B. Occurrence of hatching

Fertilization site	Peptide	120 h post-hCG		144 h post-hCG		
		Blastocysts (*n*)	Hatching (*n*)	Blastocysts (*n*)	Hatching (*n*)	Hatched (*n*)
In vitro	Gln	56	14 (25.0%)[c]	113	63 (55.8%)[c]	10 (8.8%)[d]
In vitro	GlyGln	71	21 (29.6%)	99	82 (82.9%)	27 (27.3%)
In vivo	Gln	57	17 (29.8%)	67	34 (50.7%)	10 (14.9%)
In vivo	GlyGln	65	12 (18.5%)	74	57 (77.0%)	28 (37.8%)
Fisher's exact test (*P*)			0.402		0	0

[a]Assumed to be an estimate of the fertilization rate.
[b]Percentage of blastocysts developing from 2-cells.
[c]Percentage of blastocysts hatching.
[d]Percentage blastocysts hatched.

in vitro either partially or completely hatched when compared with *in vivo* control embryos. Within each group a higher proportion of blastocysts partially or completely hatched in KSOM$_g^{AA}$ when Gln is replaced with GlyGln (Table IIIB) ($P < 0.001$ in all cases).

Blastocyst cell counts

Figure 1A shows the distributions of the cell counts in the ICM of blastocysts from *in vivo* (control) and *in vitro*-derived zygotes cultured in Gln-KSOM$_g^{AA}$ and GlyGln-KSOM$_g^{AA}$. The medians of the distribution of ICM cell counts produced in the presence of GlyGln are larger than the medians of the distributions of the ICM cell counts obtained with Gln. In the *in vivo* (control) group, the notch of the distribution of ICM cell counts produced in the presence of GlyGln does not overlap with the notch of the distribution of the ICM cell counts produced in Gln. There is thus a significantly higher cell count in the ICM of blastocysts derived from culture in GlyGln-KSOM$_g^{AA}$. In the blastocysts derived from zygotes fertilized *in vitro*, the notches of the distributions of the ICM cell counts in blastocysts produced in the presence of GlyGln and Gln slightly overlap. Thus, the difference just fails to reach statistical significance. Nevertheless the ANOVA (data not shown) of the logarithms of the ICM cell counts, in which the variation between replicates is eliminated, demonstrate a statistically significant ($P = 0.002$) effect of replacing Gln with GlyGln in KSOM$_g^{AA}$.

Figure 1B shows the distribution of the cell counts in the TE of blastocysts derived from control (*in vivo*) and *in vitro* derived zygotes cultured in Gln-KSOM$_g^{AA}$ and GlyGln-KSOM$_g^{AA}$. The cell counts vary over a wide range in all experimental groups. The notches of the four distributions all overlap, so there are no statistical differences between the median cell counts using Gln and GlyGln or the two methods of fertilization. Nevertheless, the TE cell counts in blastocysts produced using GlyGln are slightly larger than those using Gln in both the *in vitro* and in *vivo* groups. The ANOVA (data not shown) of the logarithms of the TE cell counts, which eliminates the variation between replicates, failed to show a statistically significant effect of replacing

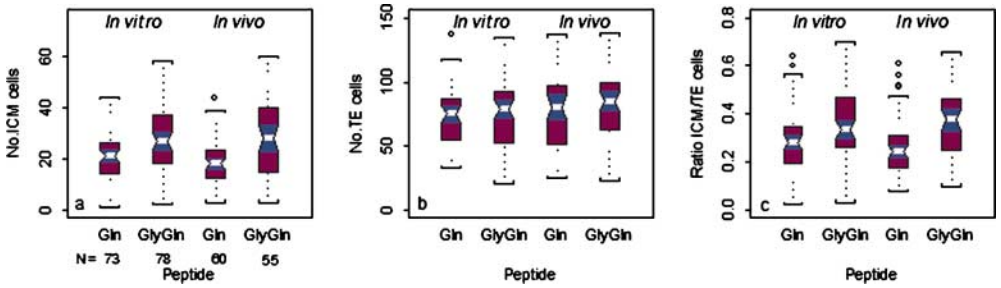

Figure 1. Distributions, summarized as notched box plots, of the numbers of ICM (A), and TE (B) in blastocysts cultured for 144 h post-hCG administration in KSOM$_g^{AA}$ supplemented with either 2 mmol/l Gln or GlyGln. Zygotes were derived from fertilization *in vitro* (IVF) and from *in vivo* (control). (C) The distributions, summarized as notched box plots, of the ratio of ICM to TE cells. The box plots show the 10th, 25th, 50th (median), 75th and 90th percentiles, respectively. The values of the medians of each box plot are shown to the left of the notches.

Gln with GlyGln in $KSOM_g^{AA}$: fertilization *in vitro* ($P = 0.367$) and *in vivo* controls ($P = 0.423$).

Figure 1C shows the distributions of the ratio of the numbers of ICM and TE cells in blastocysts produced from control (*in vivo*) and *in vitro*-derived zygotes cultured in the presence of Gln-$KSOM_g^{AA}$ and GlyGln-$KSOM_g^{AA}$. The presence of GlyGln has a greater relative stimulatory effect on the ICM cells than on the TE cells both *in vitro* and *in vivo*. For IVF-produced blastocysts the ratio of the two distributions of the ICM and TE is higher in blastocysts produced in GlyGln compared with Gln, but the notches overlap, showing that the effects of Gln and GlyGln are not significant. The ANOVAs (data not shown) of the logarithms of cell counts of the ratio of ICM/TE, which eliminates the variation between replicates, shows a statistically significant effect of replacing Gln with GlyGln in both treatment groups: fertilization *in vitro* ($P = 0.002$), and *in vivo* controls ($P = 0.00009$).

Blastocyst pyknotic and fragmented nuclei count

The distributions of the numbers of pyknotic and fragmented nuclei in blastocysts at 144 h post hCG cultured in $KSOM_g^{AA}$ supplemented with either 2 mmol/l Gln or 2 mmol/l GlyGln are displayed in Figure 2. Figure 2A and C shows the distributions for blastocysts derived *in vitro* and cultured in Gln and GlyGln, respectively. Figure 2B and D shows the corresponding distributions for blastocysts derived *in vivo*. The number of pyknotic and fragmented nuclei is very variable, ranging from 0 to 19 cells. Visual comparison of the distributions of the proportions of pyknotic and fragmented nuclei in media containing either Gln or GlyGln suggests that those associated with GlyGln are more skewed to the left. This skewness is reflected in the medians of the distributions obtained using GlyGln which are less than those using Gln. The median number of pyknotic and fragmented nuclei was five for blastocysts derived from culture in GlyGln-$KSOM_g^{AA}$ and approximately six or seven in

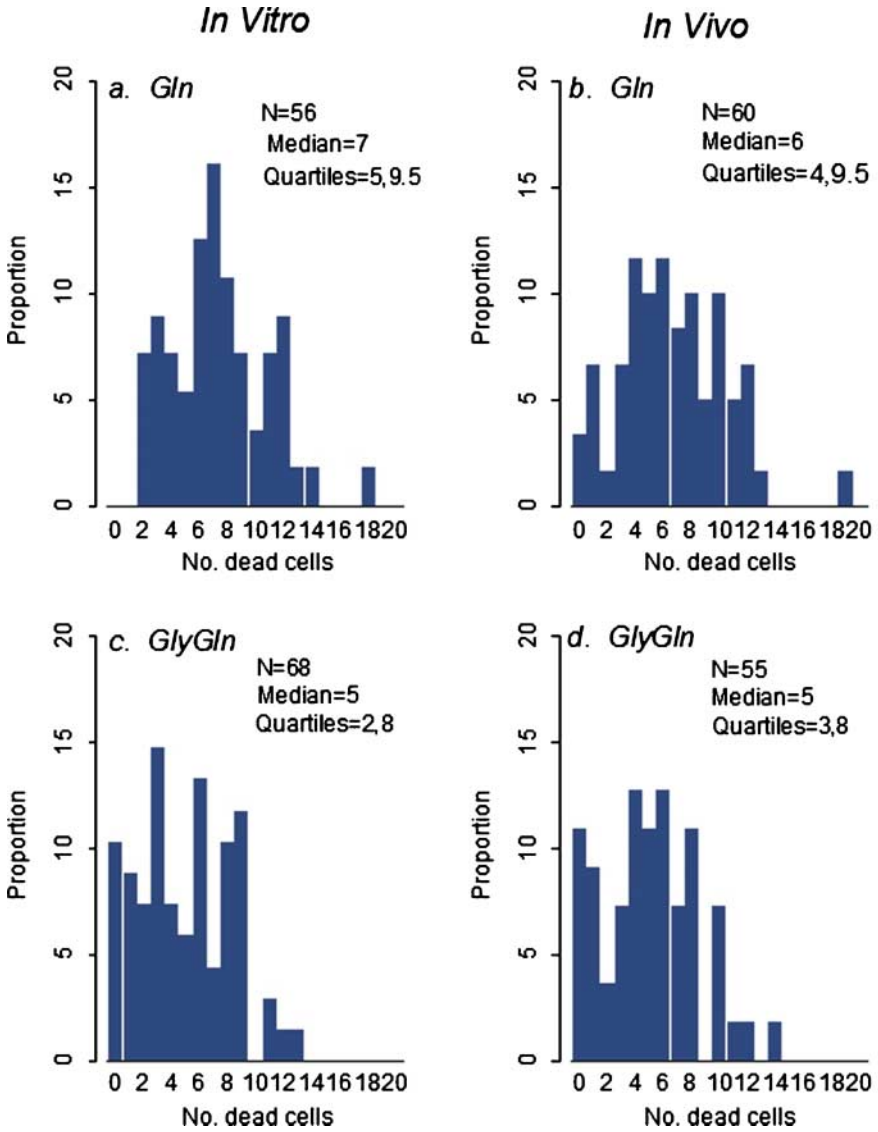

Figure 2. Barplots of the numbers of dead cells observed in blastocysts cultured from zygotes for 144 h post-hCG administration in $KSOM_g^{AA}$ supplemented with either 2 mmol/l Gln or 2 mmol/l GlyGln. Zygotes were derived from fertilization *in vitro* (IVF) and from *in vivo* (control): (A) Gln/IVF; (B) Gln/*in vivo*; (C) GlyGln/IVF; (D) GlyGln/*in vivo*.

Gln-KSOM$_g^{AA}$. An ANOVA (data not shown) demonstrated that the method of fertilization under our conditions had no effect on the numbers of pyknotic and fragmented nuclei. The only significant difference was due to effects of Gln and GlyGln.

Effect of replacing Gln with GlyGln in KSOM$_g^{AA}$ on fetal development

Compacted morulae and blastocysts for embryo transfer were obtained from *in vivo* (control) and *in vitro*-derived zygotes cultured in Gln-KSOM$_g^{AA}$ and GlyGln-KSOM$_g^{AA}$. The embryos were transferred to 22 recipients. Four of the recipients were not pregnant and three recipients had implantations and fetuses in only one uterine horn. These were not included in the statistical analysis since there is uncertainty over whether the result is due to embryonic loss or failure in the transfer process. These fetuses were included, however, in the assessment of abnormal morphology. The results of transfer to 15 recipients were used for statistical analysis: seven recipients received *in vitro*-embryos and eight received *in vivo*-embryos.

Since statistical analyses of the results using IVF showed that the data were homogeneous, they were pooled. The common odds ratios show that there is no significant difference in the incidence of implantations or fetuses that developed from zygotes produced *in vitro* using Gln or GlyGln (Table IV). Statistical analyses of the results using *in vivo* fertilization were also homogeneous and were pooled. The common odds ratios showed, in contrast, that there are significant differences in the numbers of implantations and fetuses when the zygotes were produced *in vivo* (Table IV). More implantations and fetuses were seen when embryos were cultured in Gln-KSOM$_g^{AA}$, compared with GlyGln-KSOM$_g^{AA}$.

A total of 112 fetuses were examined and body weights recorded. No gross morphological abnormalities were seen. The fetal weight data on day 14 and day 15 fetuses are summarized in Table V. There are no significant differences between the different treatment groups. The differences between the fetal body weights on day 14 and day 15 are statistically significant ($P < 10^{-5}$), reflecting the growth of the fetuses during this period. There was no difference in the interval growth of the fetuses in the different treatment groups.

Table IV. The numbers of implantations and fetuses that developed from compacted morulae and blastocysts produced from ova fertilized *in vitro* and naturally after culture to morulae and blastocysts in the presence of Gln-KSOM$_g^{AA}$ or GlyGln-KSOM$_g^{AA}$ and transfer to surrogate mothers

Fertilization site	Peptide	Transferred (*n*)	Implantations (*n*)	Fetuses (*n*)
In vitro	Gln	42	34 (81.0%)[a]	20 (47.6%)[c]
In vitro	GlyGln	42	28 (66.7%)[a]	20 (47.6%)[c]
In vivo	Gln	48	42 (87.5%)[b]	38 (79.1%)[d]
In vivo	GlyGln	48	32 (66.7%)[b]	24 (50%)[d]

[a]Implantation: *in vitro*, Gln v GlyGln: common odds ratio 0.39, 95% confidence limits 0.12–1.23, $P = 0.167$.
[b]Implantation: *in vivo*, Gln v GlyGln: common odds ratio 0.29, 95% confidence limits 0.09–0.81, $P = 0.027$.
[c]Fetuses: *in vitro*, Gln v GlyGln: common odds ratio 1.00, 95% confidence limits 0.39–2.54, $P = 1.00$.
[d]Fetuses: *in vivo*, Gln v GlyGln: common odds ratio 0.26, 95% confidence limits 0.10–0.65, $P = 0.0049$.

Table V. The body weights of fetuses that developed after transfer into surrogate mothers after the culture of fertilized ova produced by IVF or *in vivo* (control)

Fertilization site	Age (days)[a]	Peptide	*n*	Body weight (g)	SEM
In vitro	14	Gln	11	0.098	0.006
In vitro	14	GlyGln	16	0.101	0.003
In vitro	15	Gln	9	0.128	0.009
In vitro	15	GlyGln	10	0.142	0.010
In vivo	14	Gln	8	0.100	0.007
In vivo	14	GlyGln	4	0.084	0.006
In vivo	15	Gln	30	0.154	0.005
In vivo	15	GlyGln	20	0.146	0.006

[a]Post-hCG.

Discussion

The results demonstrate that exposure of ova to a medium containing Gln before and after fertilization *in vitro* resulted in the production of a large proportion of blastocysts after culture until 144 h post-hCG. Transfer of some of the blastocysts at 96 h post-hCG resulted in well-developed fetuses with body weights in the normal published range. No evidence of gross fetal abnormalities, such as exencephaly, were observed. The development of control zygotes produced by *in vivo* fertilization, followed by culture until 96 h post-hCG and transferred to surrogate mothers, developed in the same manner. No gross fetal abnormalities were observed.

Lane and Gardner (1994) cultured mouse zygotes in a medium containing 1 mmol/l Gln before transferring them to surrogate mothers. This concentration is commonly used in preimplantation culture media. Several cases of exencephaly were observed and were attributed to the accumulation of ammonium in the culture medium (Lane and Gardner, 1994). During the culture of preimplantation embryos there are several potential sources of ammonium, including amino acid and protein metabolism/turnover by the embryo, spontaneous breakdown of the amino acids, most notably Gln, and perhaps from the breakdown/metabolism of albumin in the culture media. However, the relative contributions of all other sources are proportionately very small when compared to the chemical breakdown of Gln. Nakazawa *et al.* (1997) showed that the addition of mouse embryos to their amino acid-supplemented culture media did little to increase the measured ammonium concentration. Further, Lane and Gardner (2003) demonstrated that the rates of accumulation of ammonium in two embryo culture media that were supplemented with essential and non-essential amino acids and with AlaGln replacing Gln were negligible. The estimated ammonium production of preimplantation mouse embryos at the blastocyst stage is ~1.0 pmol/embryo/h (Gardner and Lane, 1993) and 4.3 pmol/embryo/h for bovine embryos. The latter higher value is attributable to the known higher levels of metabolic activity of bovine embryos (Orsi and Leese, 2004). In our culture system, 12 mouse

embryos are cultured in 50 μl droplets of culture media. Consequently, over 5 days of culture, the ammonium generated by the metabolism of the embryos would result in a final concentration of 0.0024 mmol/l, a value that is more than two orders of magnitude less than that derived from the breakdown of Gln in aqueous solution. This estimate is probably exaggerated, since amino acid utilization and turnover is lower in preimplantation embryos prior to compaction (Brinster, 1971; Kaye *et al.*, 1982, Partridge and Leese, 1996). We therefore estimate that $<2\%$ of the total ammonium is derived from all sources, other than the chemical breakdown of Gln. The rate of breakdown of Gln in aqueous solution follows first order kinetics, described by the equation:

$$[G_t] = [G_0]e^{-kt} \quad (1)$$

where $[G_0]$ and $[G_t]$ are the concentrations of Gln at $t = 0$ and at time t in hours, and k is the rate constant. We have shown that equation 1 adequately fits the data published by Gardner and Lane (2003) who measured at different times the concentrations of ammonium accumulating in $KSOM^{AA}$ containing 1 mmol/l Gln at 37 °C. The estimated rate constant was $0.0045\,h^{-1}$ and the estimated half-life of Gln at 37 °C in $KSOM^{AA}$ is $\sim 150\,h^{-1}$, a value that is similar to that obtained for the breakdown of Gln at 37 °C in other culture media (Tritsch and Moore, 1962; Heenemann *et al.*, 1993). In the present study, the Gln concentration was increased to 2 mmol/l in $KSOM^{AA}$, to exaggerate any effect of Gln and/or ammonium; the estimated ammonium concentration after 24, 48, 72, 96, 120 and 144 h at 37 °C is ~0.2, 0.39, 0.55, 0.70, 0.83 and 0.95 mmol/l, respectively.

In our studies, the culture media are equilibrated overnight at 37 °C. Thus, at time zero, the start of the fertilization process *in vitro*, it is estimated that the medium contains 0.14 mmol/l ammonium. At the completion of fertilization, the ammonium concentration is expected to rise to 0.18 mmol/l. The zygotes were then transferred to fresh media containing ~0.14 mmol/l ammonia. By 96 h post-hCG, when blastocysts were transferred to surrogate mothers, the ammonium will accumulate to 0.70 mmol/l, and by 144 h post-hCG, when blastocyst cell counts were done, it is expected to be 0.95 mmol/l. Our results show that these gradually increasing concentrations of ammonium do not adversely affect the development of the pre- and post-natally developing mouse embryo. These results agree with other results published elsewhere on the effects of Gln on the development *in vitro* of mouse zygotes recovered from the oviduct (Biggers *et al.*, 2004a). Hammon *et al.* (2000) have also shown that bovine follicular fluid contains appreciable amounts of ammonium, and that exposure of bovine oocytes to ammonium (up to 0.35 mmol/l) did not affect their fertilization, subsequent cleavage or development to blastocysts or hatching blastocysts.

In recent years, Gardner and Lane have modeled the alleged toxicity of Gln to mouse preimplantation development by studying the direct effects of added ammonium chloride to the culture medium (for review, see Lane and Gardner, 2003). In their experiments, ammonium chloride was added in high concentrations at the beginning of the culture period. Their protocols do not reproduce the conditions created by the slow accumulation of ammonium from the spontaneous breakdown of Gln, and is thus a poor model of the conditions that occur under commonly used culture conditions. For example, Lane and Gardner (2003) have previously reported that the addition of exogenous ammonium to cultures of mouse preimplantation embryos resulted in a significant decrease in an embryo's ability to result in a viable pregnancy after embryo transfer, resulting in lower implantation rates, decreased fetal growth and an increased incidence of fetal anomalies. We observed no such findings either in the present study, or previous studies (Biggers *et al.*, 2004a), likely reflecting the well known ability of embryos to undergo post-transfer compensatory growth. We did observe a higher implantation rate and fetal viability for *in vivo*-derived embryos cultured in $KSOM_g^{AA}$ containing 2 mmol/l Gln, when compared with GlyGln. This was not observed, however, in a much larger series of embryo transfer studies (Biggers *et al.*, 2004a), probably reflecting the relatively small group sizes in the present study. The problems involved with adding exogenous ammonium to embryo culture media and the reasons for the differences have been discussed in more detail elsewhere (Biggers *et al.*, 2004b).

The replacement of Gln with GlyGln in the fertilization and culture media, as expected, had no effect on the rate of development of the mouse embryos and also did not result in any gross abnormal development. Hagemann *et al.* (1998) observed no difference in fertilization rates of bovine oocytes or embryo development in media supplemented with either Gln or GlyGln.

Elsewhere, we have reported that replacing Gln with GlyGln in Gln-$KSOM_g^{AA}$ increases the numbers of cells that develop in the blastocyst from zygotes produced *in vivo*, particularly the ICM cells (Biggers *et al.*, 2004a). The results reported in the present paper using zygotes produced both *in vitro* and *in vivo* confirm this result. In addition, our results show that replacing Gln with GlyGln in Gln-$KSOM_g^{AA}$ decreases the total numbers of pyknotic and fragmented nuclei observed in the blastocyst. This method does not distinguish between cells dying of oncosis or apoptosis. The median number of pyknotic and fragmented nuclei in blastocysts was not affected by the method of fertilization when cultured in GlyGln-$KSOM_g^{AA}$ (median = 5). Statistically significant higher median pyknotic and fragmented nuclei counts were seen in blastocysts fertilized and cultured in Gln-$KSOM_g^{AA}$ (*in vitro*: median = 7; *in vivo*: median = 6). Whether the higher numbers of pyknotic and fragmented nuclei in blastocysts produced in Gln-$KSOM_g^{AA}$ is due to the breakdown of Gln is unknown. Kamjoo *et al.* (2002) reported that the level of apoptosis in the ICM of mouse embryos was dependent on the culture media used and the choice of mouse strain.

Although there is no evidence that fertilization *in vitro* in the presence of Gln is detrimental to later development, there may be an advantage of replacing Gln with GlyGln because of the stimulation of the development of the ICM and the

reduction in the number of dead cells. Elsewhere, we have presented evidence that AlaGln, now being added to several commercial media to overcome the accumulation of ammonium in preimplantation culture media, does not have the stimulatory effect on the ICM as GlyGln (Biggers, 2004a).

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