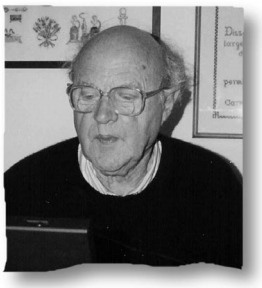
Article

Enhanced effect of glycyl-L-glutamine on mouse preimplantation embryos *in vitro*



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Abstract

A comparison of the effects of replacing L-glutamine with either glycyl-L-glutamine or alanyl-L-glutamine in a KSOM-type medium on the development of mouse preimplantation embryos *in vitro* has been made. Alanyl-L-glutamine has no significant effect on the rates of blastocyst formation, onset or completion of hatching, and on the numbers of inner cell mass and trophectoderm cells that develop. Glycyl-L-glutamine has no effect on the rate of blastocyst formation; it stimulates slightly the onset of hatching, but significantly increases the numbers of inner cell mass and trophectoderm cells that develop. Embryo transfer experiments comparing media containing either glutamine or glycyl-L-glutamine have not produced any gross abnormal fetal development. Recently, alanyl-L-glutamine has been used to replace glutamine in media for the culture of human preimplantation embryos. The results in this paper suggest that glycyl-L-glutamine may be a better choice of dipeptide.

Keywords: *dipeptides, embryo culture, exencephaly, glutamine*

Introduction

The toxicity of glutamine (Gln) in culture media is well known (Visek *et al*., 1972; Ito and McLimans, 1981) and is attributed to the accumulation of ammonium that arises from its breakdown or metabolism (McLimans *et al*., 1981; Heeneman *et al*., 1993). In the absence of living tissue, ammonium forms by the spontaneous breakdown of Gln into ammonia and 2-pyrrolidone-5-carboxylic acid (non-metabolic ammonia) (Vickery *et al*., 1935). When tissue is present, ammonium also forms due to the metabolism of Gln (Newsholme and Newsholme, 1989) and other metabolites (metabolic ammonia). The accumulation of ammonium and 2-pyrrolidone-5-carboxylic acid can be reduced by substituting more stable dipeptides that can also be utilized by cells *in vitro* (Eagle, 1955). The suggestion has been made that these dipeptides are first hydrolysed extracellularly by a peptidase secreted by the cultured cells (Christie and Butler, 1994). Roth *et al*. (1988) used alanyl-L-glutamine (AlaGln) or glycyl-L-glutamine (GlyGln) to prevent the deleterious effect of autoclaving medium RPMI, which contains glutamine, on the growth of K562 cells. Since then, these glutamine-containing dipeptides have been used to replace glutamine in media employed in the culture of other cell types (Brand *et al*., 1989; Bulus *et al*., 1989; Holmlund *et al*., 1992; Christie and Butler, 1994). Subsequently, Atanassov *et al*. (1998) showed that the concentration of AlaGln in cell cultures needs to be optimized using concentration response studies.

Interest in the possible toxicity of Gln in media used for the culture of mammalian preimplantation embryos arose from the work of Lane and Gardner (1994), who suggested that the cases of exencephaly observed in mice was due to ammonium produced from Gln in the culture media they used. Elsewhere, the interpretation of their work has been questioned (Biggers *et al*., 2004). There appears to be no evidence that the concentrations of Gln used in preimplantation culture media is toxic to the preimplantation embryo of any other species, including the human embryo. Nevertheless, several commercially available media for the culture of human

preimplantation embryos now include AlaGln instead of Gln to reduce the accumulation of toxic ammonium, although there has been no definitive investigation of the effects of this dipeptide on the preimplantation embryo *in vitro* using concentration response studies (e.g. G-1™ version 3 medium; Vitrolife, Göteborg, Sweden; Global®; LifeGlobal, Canada; Quinn's Advantage® cleavage medium; Cooper Surgical, Trumbull, CT, USA). Lane *et al*. (2001) substituted AlnGln for Gln in a modified G1.2 medium for the culture of mouse preimplantation embryos using only one concentration, while simultaneously varying the concentrations of all the other amino acids. An earlier study reported no differences between the development of bovine embryos in a modified SOF medium containing either Gln or GlyGln (Hagemann *et al*., 1998), again using only one concentration. No investigation has been made of the possibility that all dipeptides do not have equivalent effects. The present study now reports on the effects of replacing Gln in medium KSOM supplemented with all the other 19 natural amino acids with various concentrations of AlaGln or GlyGln on the development of mouse embryos, including evidence that the effects of dipeptides may vary.

Materials and methods

Media

Several media have been used in this work that are based on medium $mKSOM_{g/BSA}^{AA}$ shown in **Table 1**. This medium is a modification of medium KSOM (Lawitts and Biggers, 1993) in which the concentration of glucose has been raised to 5.56 mmol/l, together with the addition of the 19 amino acids. The medium differs from a similar one called mKSOM (Summers *et al*., 1995). In one experiment, the BSA was replaced with polyvinyl alcohol (PVA) in a concentration of 1 mg/ml (Biggers *et al*., 1997, 2000), resulting in the media denoted $mKSOM_{g/PVA}$ and $mKSOM_{g/PVA}^{AA}$. Note that the prefix denotes that the medium is a modification of the original KSOM, the subscripted suffixes denote specific modifications or substitutions of the original KSOM and the superscripted suffixes denote additions to the original KSOM.

Donors

Virgin CF1 female mice, and 2 to 11-month-old C57BL/6J × DBA/2J (BDF) male mice (Charles River Laboratories, Raleigh, NC, USA) were used in this study. Animals were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of Harvard Medical School. The female mice were superstimulated with 5 IU pregnant mares' serum gonadotrophin (ECG), and superovulated with 5 IU human chorionic gonadotrophin (HCG) 48 h later, after which they were mated to BDF males. The outbred one-cell BDF *male* × CF1 *female* embryos were collected from the oviduct 22–26 h post-HCG. Those with two pronuclei were selected for culture. A modified flushing-holding medium (FHM) (Lawitts and Biggers, 1993), in which the BSA was replaced with 1.0 mg/ml PVA, was used for oviduct flushing, embryo collection and holding prior to culture.

Table 1. Composition of $mKSOM_{g/BSA}^{AA}$ used as the base medium.

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
Glutamine	1
EDTA	0.01
BSA	1.0[a]
L-Alanine-HCl	0.05
L-Arginine-HCl	0.05
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

[a]mg/ml.

Preparation of culture media

All culture media were formulated from KSOM using the procedures described elsewhere (Biggers *et al*., 2000). Eagle's mixtures of essential and non-essential AA were purchased from Gibco BRL (Life Technologies, Inc. Grand Island, NY, USA). ECG and HCG and all other chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA).

Embryo culture

Embryos were cultured in groups of 12 per 50 µl droplet of medium overlaid with embryo-tested mineral oil. Embryos were cultured in modular incubator chambers (Billups Rothenberg Inc., Del Mar, CA, USA) which were gassed with a mixture of 5% O_2, 6% CO_2 and 89% N_2. The pH of all equilibrated media was approximately 7.3. Culture plates (60 mm suspension dish; Corning Inc., Corning, NY, USA) were prepared 1 day before embryo collection and equilibrated in the module overnight. Embryos were cultured for 5 days (144 h post-HCG).

Embryo evaluation

Embryos were observed 96, 120 and 144 h post-HCG at ×40 magnification on a warmed microscope stage at approximately 35°C (Wild dissecting microscope), and graded for the stage of development including compaction, blastocoel formation and hatching. These times correspond approximately to 72, 96 and 120 h in culture.

Differential ICM and TE cell counts

After 144 h of culture, blastocysts were stained with polynucleotide-specific fluorochromes to differentially stain ICM and TE cells using a modification of a method originally described by Handyside and Hunter (1984, 1986) and Papaioannou and Ebert (1988). The details of the modified method are described by Biggers *et al*. (2000).

Embryo transfer

Pseudopregnant CD1 female mice were produced by mating CD1 females to vasectomized CD1 males. Cultured blastocysts and compact morulae (day 4 of culture) were transferred into day 3 pseudopregnant females. Fetuses were collected from the pregnant mice on day 12 after transfer for gross examination and body weight measurements.

Biometrical methods

The results of four experiments are described in this paper; three embryo culture experiments and one embryo transfer experiment. Experiment 1 was a randomized block experiment with the treatments in a 3 × 3 factorial array that compared the effects of Gln, AlaGln or GlyGln at three concentrations in $mKSOM_{g/BSA}^{AA}$. Experiment 2 was a randomized block experiment with the treatments in a 2 × 5 array which compared the effects of Gln and GlyGln over a wider range of five concentrations in $mKSOM_{g/BSA}^{AA}$, and also included a group of cultures where Gln and GlyGln were omitted. Experiment 3 was a randomized block experiment with three treatments comparing the effects of Gln and GylGln in $mKSOM_{g/PVA}^{AA}$. A group of 12 zygotes formed the experimental unit in all experiments, but the numbers of units in each cell of the experimental design were sometimes unequal.

Two sets of observations were made in these three experiments. Set 1 concerned the morphological development of the zygotes into 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 develop into blastocysts, that at least start to hatch, and that at least completely hatch. These categorical observations are longitudinal in both time and in a developmental sequence. They are summarized graphically as a matrix of graphs to display these longitudinal relations simultaneously. These categorical data were analysed using a parametric generalized linear model (McCullagh and Nelder, 1989), assuming that the errors follow a binomial distribution. Set 2 concerns the numbers 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 bar charts or notched box plots. The boxplots show the 10th, 25th, 50th (median), 75th and 90th percentiles; observations outside the 10th and 90th percentiles are considered outliers. The notches on the boxplots are the 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 assuming log normally distributed errors.

Experiment 4 compared the effects of Gln and GlyGln on fetal development after transfer of blastocysts into surrogate mothers. In this experiment, a set of up to six embryos cultured in Gln containing $KSOM_{g/PVA}^{AA}$ was injected into the left or right uterine horn at random and another set of embryos cultured in GlyGln-containing $KSOM_{g/PVA}^{AA}$ was injected into the contralateral uterine horn. The numbers of implantation sites and fetuses were counted 12 days after the embryo transfers were done. The data were treated as a set of stratified 2 × 2 contingency tables (Mehta and Patel, 2001), each stratum being one mother. A few recipients had implantations and fetuses in only one uterine horn. These cases were excluded from the analysis since there is ambiguity between whether the result is due to embryonic loss or failure in the transfer process. The exact probability that the sets were homogenous was calculated. If $P > 0.05$ the pooled exact estimate of the common odds ratio and its confidence limits ($P = 0.95$) was computed. The effects of the two media were considered significantly different if the confidence limits did not include 1. Also Spearman's rank correlation coefficients between the numbers of ICM and TE cells were computed (Sheskin, 2000).

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

Results

Comparison of the development of zygotes into blastocysts in $mKSOM_{g/BSA}^{AA}$ containing different concentrations of Gln, AlaGln or GlyGln

The development of mouse zygotes into blastocysts was compared over a culture period lasting 144 h in $mKSOM_{g/PVA}^{AA}$ containing three concentrations (1, 2, 3 mmol/l) of Gln, AlaGln or GlyGln. Three replicates were performed, having one, two and two experimental units per treatment, respectively.

Morphological responses

The numbers of zygotes that developed into blastocysts, initiated hatching and completed hatching at 96, 120 and 144 h post-HCG were recorded. Analyses of deviance were performed on all sets of data where responses occurred. In all cases there were significant differences between replicates, but

no significant two-way interactions between replicates and the experimental factors (peptides and concentration). Thus, in all cases, the effects of peptide and concentration on the percentages of zygotes that developed at least to blastocysts, initiated hatching and completed hatching at 96, 120 and 144 h post-HCG have been pooled over replicates. The results are summarized in **Figure 1a–i**.

Only the analyses of deviance on the data obtained 144 h post-HCG are shown in **Table 2**, as the analyses on the data obtained at 96 and 120 h post-HCG offer nothing of unique significance. The analysis of deviance on the numbers of zygotes that developed at least into blastocysts by 144 h post-HCG shows that the two treatment main effects (peptides and concentration) and the interaction between them were not significant (**Table 2**). Thus, there was no significant difference in the percentages of blastocysts that developed. Pooling these results, 77% of the embryos were blastocysts by 144 h post-HCG. A second analysis of deviance demonstrated a significant difference between the effects of peptides on the percentages of blastocysts that started to hatch in media containing Gln, AlaGln or GlyGln at 144 h post-HCG (P = 0.015), and also a significant peptide × concentration interaction (P = 0.015) (**Table 2**). The percentage of blastocysts that started to hatch was slightly inhibited by raising the concentration of Gln (**Figure 1f**). In contrast, the addition of AlaGln or GlyGln had no inhibitory effect on the percentage of blastocysts that started to hatch. A third analysis of deviance demonstrated a significant difference between the effects of peptides on the percentages of blastocysts that started to hatch in media containing Gln, AlaGln or GlyGln at 144 h post-HCG (P = 0.012), but no significant peptide × concentration interaction (**Table 2**). The results in **Figure 1i** suggest the effect is due to slightly higher incidence of complete hatching in the presence of GlyGln.

Cell counts

The analyses of variance (ANOVA) (not shown), which eliminate the large variation between replicates, show that the

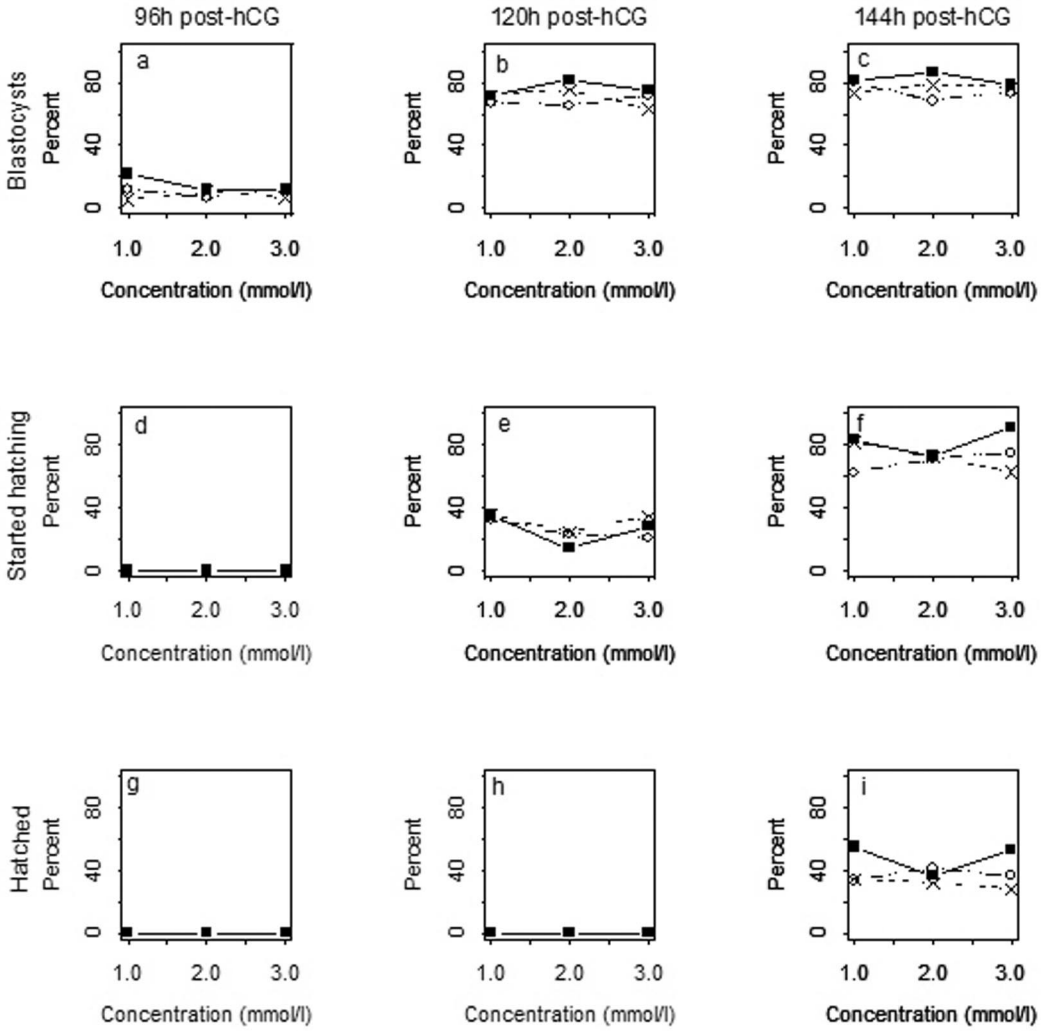

Figure 1. A response matrix showing the percentage of zygotes that develop into blastocysts, the percentage of blastocysts that commence hatching, and the percentage of blastocysts that complete hatching when cultured in $mKSOM_{BSA}^{AA}$ containing different concentrations of Gln, AlaGln or GlyGln for 96, 120 and 144 h post-HCG. Crosses = Gln; open circles = AlaGln; solid squares = GlyGln.

Table 2. Analyses of deviance of the percentages of zygotes that developed at least into blastocysts by 144 h post-HCG in $mKSOM_{g/BSA}^{AA}$, and the percentages of blastocysts that started hatching and completed hatching by 144 h post-HCG.

Variation	*df*	*At least to blastocyst*		*Started hatching*		*Completed hatching*	
		Deviance	P-value	*Deviance*	P-value	*Deviance*	P-value
Replicates (R)	2	56.231	$<10^{-5}$	15.731	0.0004	22.585	0.00001
Peptide (P)	2	4.356	0.113	8.394	0.015	8.802	0.012
Concentration (C)	2	0.308	0.857	0.78	0.677	0.644	0.725
P × C	4	3.81	0.432	12.335	0.015	5.973	0.201
R × P	4	2.981	0.561	5.68	0.224	3.442	0.487
R × C	4	3.303	0.508	2.604	0.626	3.92	0.417
R × P × C	8	12.832	0.118	13.461	0.097	11.961	0.153
Residual	18	26.58	0.087	16.301	0.572	16.579	0.552

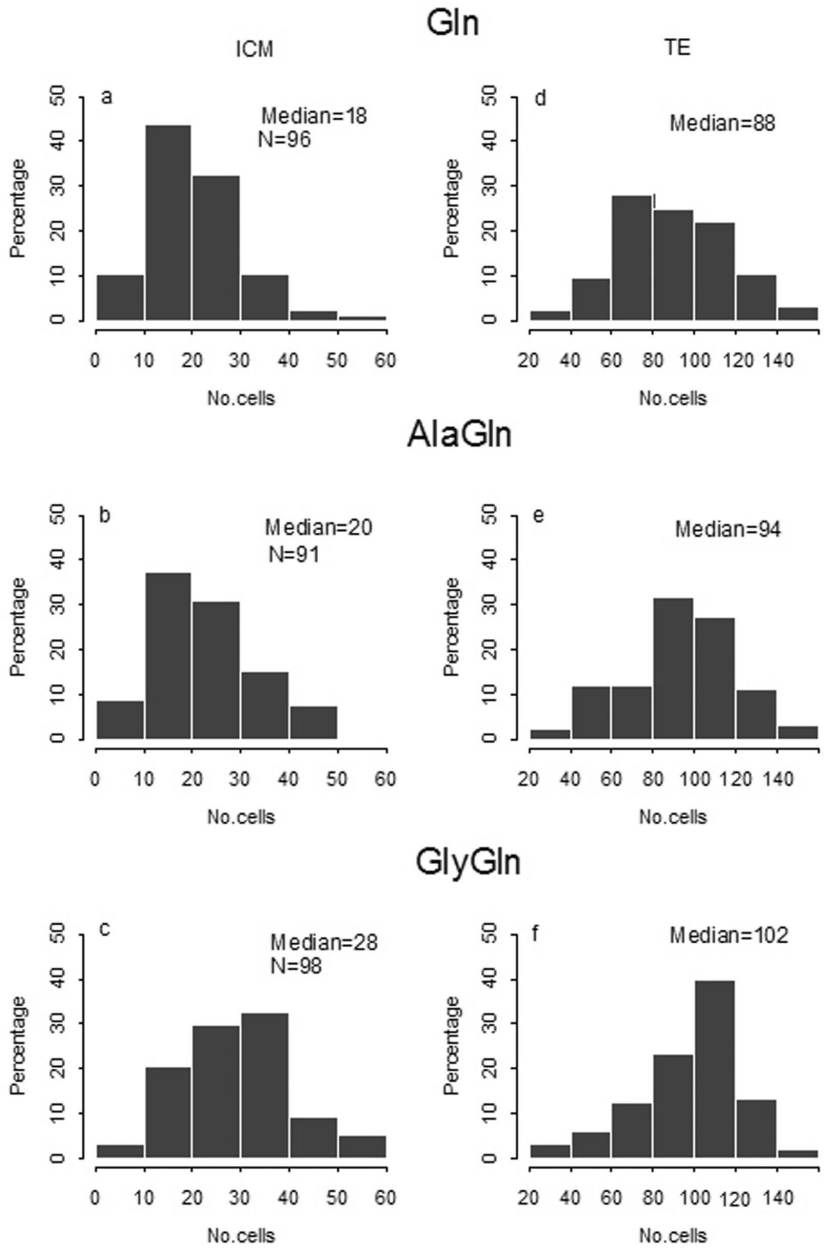

Figure 2. The distributions of the numbers of ICM and TE cells in blastocysts produced from zygotes cultured in $mKSOM_{BSA}^{AA}$ containing Gln (**a**,**d**), AlaGln (**b**,**e**) or GlyGln (**c**,**f**).

differences between the effects of the media on the numbers of ICM cells are highly significant ($P < 10^{-5}$), whereas the effects on the numbers of TE cells is only marginally significant ($P = 0.043$). The effects of varying the concentrations of the peptides on the numbers of ICM and TE cells are not significant, nor are the peptide × concentrations interactions. Thus, in the subsequent analysis, the cell counts have been pooled over the peptide concentrations.

The distributions of the pooled cell counts in the ICM and TE of blastocysts cultivated from zygotes in $mKSOM_{g/BSA}^{AA}$ containing Gln, AlaGln or GlyGln, respectively, are shown in **Figure 2a–f**. The numbers of cells in the ICM vary over a wide range (Gln: 3–50; AlaGln: 3–49; GlyGln: 3–57). The shapes of the distributions of the cell counts in blastocysts produced in the presence of Gln and AlaGln are skewed to the left and follow the Poisson type of distribution found in cell count data. In contrast, the shape of the distribution of the numbers of ICM cells in blastocysts produced in the presence of GlyGln is skewed to the right. The difference is due to more ICM cells being produced in the presence of GlyGln. The numbers of cells in the TE also vary over a wide range (Gln: 27–156; AlaGln: 30–153; GlyGln: 35–148). The shapes of the distributions of the cell counts of blastocysts that developed in the presence of Gln is skewed to the left, whereas the distributions of the counts produced in the presence of AlaGln and particularly GlyGln are skewed to the right. The differences are due to increases in the numbers of cells produced in the TE, slightly in AlaGln and markedly in GlyGln. These differences in cell counts are reflected in the medians shown in **Figure 2a–c**.

Despite the wide variations in the numbers of ICM and TE cells in the blastocysts produced in the presence of either of the three peptides, the paired observations on each blastocyst are significantly related (Gln: $r = 0.389$, $n = 96$, $P < 0.001$; AlaGln: $r = 0.466$, $n = 91$, $P < 0.001$; GlyGln: $r = 0.443$, $n = 98$, $P < 0.001$). The ratio of the numbers of ICM and TE cells in each blastocyst have therefore been separately calculated. An ANOVA (**Table 3**) shows a significant effect of peptides. The distributions of these ratios for each peptide are shown as box plots in **Figure 3**. The medians of the ratios, appended to the box plots, observed using Gln and AlaGln are very similar, whereas the median observed using GlyGln is higher.

Comparison of the effects of lower concentrations of Gln and GlyGln on the development of zygotes into blastocysts in $mKSOM_{g/BSA}^{AA}$

The effects of four concentrations of Gln and GlyGln (0.25, 0.5, 1, 2 mmol/l) were compared in $mKSOM_{g/BSA}^{AA}$. A control medium containing no glutamine was included. Lower concentrations were used since the previous experiment suggested that a concentration of 1 mmol/l Gln or GlyGln resulted in a maximal rate of blastocyst formation. Four replicates were performed with two experimental units per treatment.

Morphological responses

The overall results were similar to those summarized in **Figure 1**, so only the data obtained 144 h post-HCG are presented in an abbreviated form. The analyses of deviance show highly significant differences between replicates, but no significant interactions between replicates and the treatments (medium and concentration). Thus, the effects of medium and concentration on the percentage of zygotes developing at least to blastocysts, starting to hatch and completely hatching, have been pooled over replicates (**Table 4**).

The analysis of deviance on the percentage of blastocysts that developed by 144 h post-HCG shows no statistically significant main effects of peptides and concentration. Thus, increasing the concentrations of Gln or GlyGln from 0.25 to 2 mmol/l did not affect the percentage of zygotes developing at least to the blastocyst stage, a mean of 76%, a value very close to that found in the first experiment. The analysis of deviance just fails to demonstrate statistically significant differences between the main effects of media on the percentage of blastocysts that start to hatch by 144 h post-HCG. The media × concentration interaction was not significant. There is an indication, however, that a concentration of 2 mmol/l Gln slightly inhibits the incidence of the onset of hatching, whereas a similar concentration of GlyGln does not. Lower concentrations of Gln were not inhibitory. The analysis of deviance shows a significant effect of peptides on the percentage of blastocysts that complete hatching, but no peptide × concentration interaction. Overall, the replacement of Gln with GlyGln slightly increases the percentage of blastocysts that completely hatch. The results also show that high percentages of blastocysts develop and start to hatch even when Gln is omitted from the medium.

Cell counts

ANOVA (not shown) on the logarithms of the numbers of ICM and TE cells and the ratios between them demonstrated highly significant differences between replicates, while all replicate × treatments interactions were not significant. Also, the regressions of cell numbers on concentrations were not significant. The data have therefore been pooled over replicates and concentrations and the effects of media summarized as boxplots (**Figure 4**). Omitted from the data are five trophoblastic vesicles that contained no ICM cells and only 23–88 TE cells.

No significant differences in the numbers of ICM and TE cells were found in blastocysts that developed with or without Gln in the medium. The substitution of Gln with GlyGln, however, significantly increased the medians of ICM cells from 20 to 27, but the increase in the medians of TE cells from 113 to 120 was not statistically significant. The ratio between the numbers of ICM and TE cells increased significantly when GlyGln was used in the medium instead of Gln. **Figure 4** also summarizes the distributions of the ICM and TE cell counts in blastocysts that developed from a limited number of zygotes in the Gln-free medium. The median ICM and TE cell counts were 22 and 118 respectively, which do not differ significantly from the median counts in blastocysts that developed when Gln was included in the medium.

Table 3. Development of mouse zygotes 144 h post-HCG in $mKSOM_{g/BSA}^{AA}$ containing several concentrations of Gln or GlyGln. Numbers in parentheses are percentages.

Concentration (mmol/l)	*At least to blastocysts*[a]		*At least started to hatch*[b]		*Completed hatching*[c]	
	Gln	*GlyGln*	*Gln*	*GlyGln*	*Gln*	*GlyGln*
0	73/96 (76)	–	59/73 (81)	–	12/73 (16)	–
0.25	75/96 (78)	63/96 (66)	67/75 (89)	54/63 (86)	14/75 (19)	21/63 (33)
0.5	73/96 (76)	74/96 (77)	62/73 (85)	63/74 (85)	18/73 (25)	23/74 (31)
1	70/96 (73)	84/96 (88)	57/70 (81)	75/84 (89)	12/70 (17)	18/84 (21)
2	74/96 (77)	73/96 (76)	52/74 (70)	64/73 (88)	10/74 (14)	17/73 (23)

[a]No. zygotes developing at least to blastocysts/no. zygotes (%).
[b]No. blastocysts at least starting to hatch/no. blastocysts (%).
[c]No. completely hatched blastocysts/no. blastocysts (%).

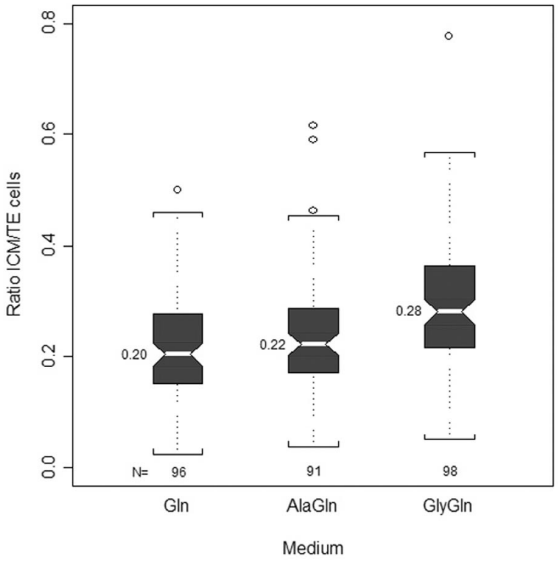

Figure 3. The distributions, summarized as boxplots, of the ratios between the numbers of ICM and TE cells in blastocysts produced from zygotes cultured in $mKSOM_{BSA}^{AA}$ containing Gln, AlaGln or GlyGln. The medians are significantly different when the notches do not overlap.

Table 4. Development of mouse zygotes in $mKSOM_{g/PVA}^{AA}$ containing no Gln, Gln or GlyGln.

Peptide	*At least to blastocysts*[a]	*At least started to hatch*[b]	*Completed hatching*[c]
None	89.0 (64/72)	94 (60/64)	44 (28/64)
Gln	87.5 (63/72)	79 (50/63)	32 (20/63)
GlyGln	87.5 (63/72)	87 (55/63)	51 (32/63)

[a]No. zygotes developing at least to blastocysts/no. zygotes(%).
[b]No. blastocysts at least starting to hatch/no. blastocysts(%).
[c]No. completely hatched blastocysts/no. blastocysts(%).

Effects of Gln and GlyGln, with and without AA, on the development of zygotes into blastocysts in $mKSOM_{g/PVA}^{AA}$

Biggers *et al*. (1997) demonstrated that the presence of BSA in media could exert a small, stimulus on the effects of AA on the development of mouse preimplantation embryos in a KSOM-type medium. The effects of Gln and GlyGln in $mKSOM_{g/PVA}^{AA}$ in which the BSA has been replaced with PVA have therefore not been examined. The development of mouse zygotes into blastocysts until 144 h post-HCG in $mKSOM_{g/PVA}^{AA}$ containing 0 and 1 mmol/l Gln and 1 mmol/l GlyGln were compared. Three replicates were performed each with two experimental units per treatment.

Morphological responses

The percentages of blastocysts that developed at least to blastocysts, that at least started to hatch and that completely hatched by 144 h post-HCG are shown in **Table 4**. The results are pooled over the three replicates, since statistical tests of the odds ratios for all comparisons done showed that the data could be assumed to be homogeneous. The percentages of zygotes that developed at least into blastocysts in the presence or absence of Gln or GlyGln were not significantly different (~88%). Thus a large percentage of blastocysts developed even if Gln was omitted. The percentage of blastocysts that started to hatch, however, was significantly lower in the medium containing Gln compared with the Gln-free medium [common odds ratio, 95% confidence limits: 0.26 (0.07–0.83)]. In contrast there was no significant difference in the rates of at least starting to hatch in the Gln-free medium and the medium containing GlyGln [common odds ratio, 95% confidence limits: 0.45 (0.11–1.60)]. Although the rate of complete hatching appears to be reduced in the medium containing Gln compared with the Gln-free medium, the difference is not significant. Similarly, the apparent increase in the rate of complete hatching in the presence of GlyGln compared with the Gln-free medium is not significant.

Cell counts

ANOVA (not shown) on the logarithms of the numbers of ICM and TE cells and the ratios between them demonstrated highly significant differences between replicates and no replicate × treatments interactions. The data from the three replicates have therefore been pooled. The distributions of the ICM and TE cell counts cultured without Gln, with Gln or GlyGln are shown as boxplots in **Figure 5**. Blastocysts with high numbers of ICM and TE cells developed in Gln-free $mKSOM_{g/PVA}^{AA}$ (**Figures 5a,b**). When Gln was included in the medium, fewer ICM cells developed while the numbers of TE cells were not affected. The numbers of ICM and TE cells that developed when GlyGln was in the medium was significantly greater than in the media that contained Gln or were Gln-free. The ratio was significantly higher when GlyGln replaced Gln in $mKSOM_{g/PVA}^{AA}$ (**Figure 5c**).

Effect of replacing Gln with GlyGln in $mKSOM_{g/BSA}^{AA}$ on fetal development

Blastocysts and compacted morulae for embryo transfer were obtained from zygotes cultured for 96 h post-HCG in $mKSOM_{g/BSA}^{AA}$ containing 2 mmol/l Gln or in $mKSOM_{g/BSA}^{AA}$ containing 2 mmol/l GlyGln. The embryos were transferred to 27 recipients. A total of 105 and 94 fetuses developed in the presence of Gln or GlyGln respectively. Only one case of exencephaly was observed and that was in the group exposed to GlyGln, an incidence of 0.74%.

Five recipients were excluded from the statistical analysis, since they had implantations or fetuses developing in only one uterine horn, leaving the data from 22 recipients for statistical analyses. The results are tabulated in **Table 5**. The analyses showed that the data from the recipients formed homogeneous groups and can be pooled. The common odds ratios show that there are no significant differences in the incidence of implantations and fetuses that developed in embryos cultured in the two media. The distributions of the body weights of the fetuses are summarized in **Figure 6**. There were no significant differences between the two groups.

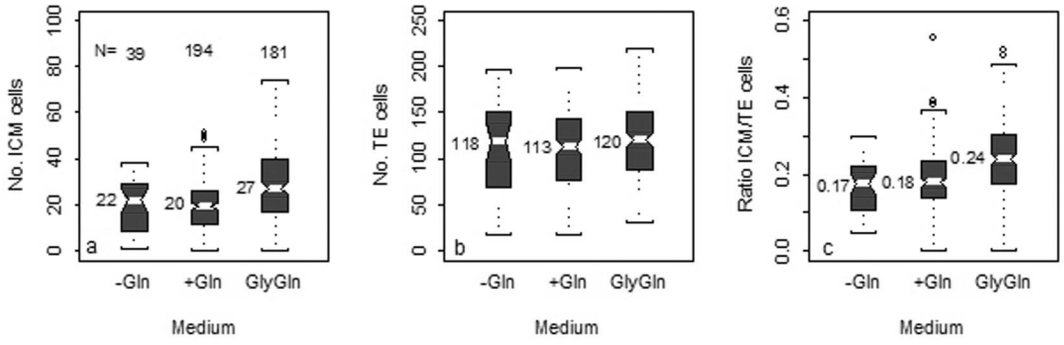

Figure 4. (**a**,**b**) The distributions, summarized as boxplots, of the numbers of ICM and TE cells in the blastocysts cultured in $mKSOM_{BSA}^{AA}$ for 144 h post-HCG lacking Gln, containing either Gln or GlyGln respectively. (**c**) Distributions of the ratio of ICM to TE cells.

Table 5. Numbers of embryos that at least implanted and developed into fetuses 12 days post-transfer produced by culture in either $mKSOM_{g/BSA}^{AA}$ containing 2 mmol/l Gln or $mKSOM_{g/BSA}^{AA}$ in which Gln was replaced by 2 mmol/l GlyGln. Values in parentheses are percentages.

Peptide	*No. recipients*	*No. embryos transferred*	*No. implantations*	*No. fetuses*
Gln	22	132	105 (79.5)	86 (65.2)
GlyGln	22	132	100 (75.8)	91[a] (68.9)

[a]One fetus had exencephaly.
Homogeneity of results of 22 mothers: Implantation data, $P = 0.261$; Fetus data, $P = 0.190$.
Common-odds-ratio tests (mid-P corrected): implantation data, 1.000 (95% confidence interval 0.542–1.845); fetus data, 1.216 (95% confidence interval 0.700–2.120).

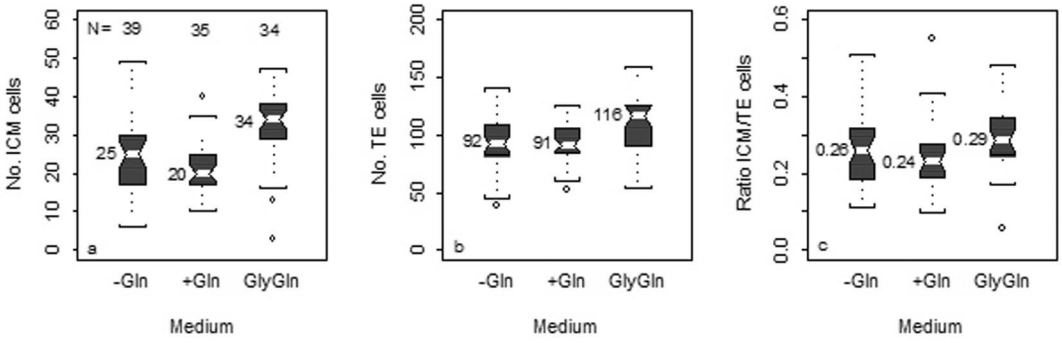

Figure 5. (**a**,**b**,**c**) The distributions, summarized as boxplots, of the numbers of ICM and TE cells, and their ratios, of blastocysts cultured in $mKSOM_{PVA}^{AA}$ for 144 h post-HCG lacking Gln, containing either 1 mmol/l Gln or 1 mmol/l GlyGln respectively.

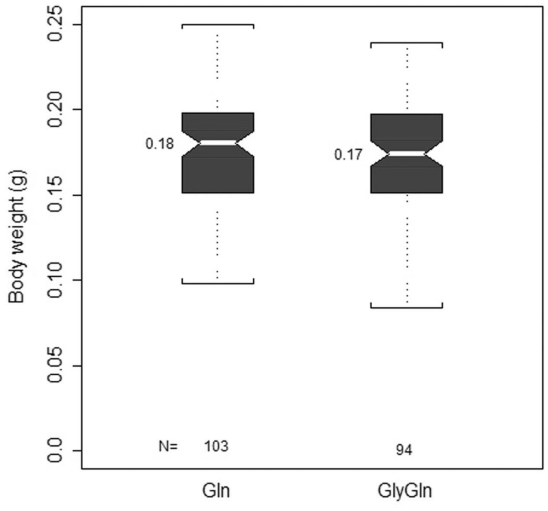

Figure 6. Distributions, summarized as boxplots, of the body weights of fetuses that developed after the transfer of embryos cultured in either $mKSOM_{BSA}^{AA}$ or $mKSOM_{BSA}^{AA}$ in which the 2 mmol/l Gln was replaced with 2 mmol/l GlyGln.

Discussion

The results obtained in experiment 1 demonstrate that mouse zygotes develop *in vitro* at similar high rates (~77%) into blastocysts when Gln is replaced with either AlaGln or GlyGln in $mKSOM_{g/BSA}^{AA}$. The blastocysts that developed started to hatch at high rates in the presence of AlaGln and GlyGln. By 144 h post-HCG, however, significantly more blastocysts completely hatched in the medium containing GlyGln compared with those cultured in the presence of Gln or AlaGln. The results also show that more ICM and TE cells developed in the medium containing GlyGln compared with the media containing Gln or AlaGln, and GlyGln caused relatively greater proliferation of the ICM cells. The results obtained in experiments 2 and 3 also confirm that the presence of GlyGln in $mKSOM_{g/BSA}^{AA}$ and $mKSOM_{g/PVA}^{AA}$ stimulates the production of cells in the blastocysts, particularly the ICM. The embryo transfer experiment (experiment 4) did not show any significant abnormal development in fetuses 12 days after transfer of blastocysts produced in GlyGln-containing $mKSOM_{g/BSA}^{AA}$. These results suggest that it is preferable to substitute GlyGln rather than AlaGln for the Gln in $mKSOM_{g/BSA}^{AA}$.

The results obtained in experiments 2 and 3 demonstrate that high yields of blastocysts develop from zygotes even in Gln-free $mKSOM_{g/BSA}^{AA}$ or Gln-free $mKSOM_{g/PVA}^{AA}$. Retaining Gln in the medium did not affect the rate of blastocyst formation. Gln, however, slightly inhibited hatching and reduced the numbers of cells that developed, particularly in the ICM of embryos cultured in $mKSOM_{g/PVA}^{AA}$. The results are partially consistent with those of Devreker and Hardy (1997), who also found no effect on the numbers of blastocysts that developed when Gln is added to Gln-free KSOM and that the blastocysts contained smaller numbers of ICM and TE cells. The results are also consistent with earlier work by Lawitts and Biggers (1992), who showed that high concentrations of glutamine (up to 3 mmol/l) inhibited blastocyst formation in the presence of low concentrations of NaCl (85 mmol/l) as in $mKSOM_{g/BSA}^{AA}$ and, and stimulate blastocyst formation when the NaCl concentration is high (125 mmol/l). Both of the current media contain relatively low concentrations of NaCl. The high yields of blastocysts when Gln is not present in the medium leave little room for the addition of AlaGln or GlyGln to show enhanced development. This condition may explain why the slopes of the observed concentration response lines were not significantly different from zero as the concentrations of AlaGln or GlyGln were increased.

When GlyGln is hydrolysed in the cell, it produces Gly and Gln in equimolar amounts. Thus, the increase in the number of cells produced in the blastocyst may depend on Gly, Gln, or both. Although Gly and Gln are both important organic osmolytes (Van Winkle *et al*., 1990; Dawson *et al*., 1998), this function cannot explain the increase in cell numbers, since the medium has a low osmolarity (~270 mosmols), and thus an osmolyte is not required. Perhaps Gln is released at an optimum rate with no possibility of ammonia or 2-pyrrolidone-5-carboxylic acid accumulating. However, the significant increase in the number of ICM cells produced by GlyGln and the lack of effect of AlaGln suggests that Gly by some unknown mechanism produced the primary effect.

The embryo transfer experiment resulted in no gross abnormalities in the fetuses that developed from preimplantation embryos cultured in the presence of Gln at twice the commonly used concentration (2 mmol/l instead of 1 mmol/l). This result is in striking contrast to the results of Lane and Gardner (1994), who reported a 20% incidence of exencephaly in fetuses that developed from blastocysts produced in mMTF, containing 1 mmol/l Gln compared with an incidence of 10% in a medium not containing Gln. The reasons for the large differences in the results from two independent laboratories are unknown. Possible reasons are discussed in an accompanying paper (Biggers *et al*., 2004). The difference in the results does question whether the presence of Gln in a medium for the culture of preimplantation embryos is necessarily teratogenic.

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