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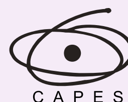

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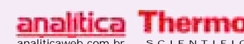

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Modulation of murine blastocyst hatching *in vitro* by glutamine and tryptophan

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Abstract

Enrichment of culture media with amino acids improves embryo development. However, little is known about the specific action of each amino acid during embryogenesis. The present study was undertaken to examine the effect of L-glutamine (Gln) and tryptophan (Trp) on mouse embryo hatching, expansion and viability *in vitro*. Blastocysts were collected from 6- to 8-week-old female BALB/c mice (N = 30) and cultured in M2 medium containing either 0.125, 0.25 or 0.5 mM Trp, 1 mM Gln, or M2 alone. Gln significantly increased (100%; $P < 0.05$) blastocyst hatching at 24 h compared to M2 alone or Trp; moreover, Trp inhibited blastocyst hatching when compared to M2 alone ($P < 0.05$) at 72 h. In contrast, the percentage of embryos reaching the state of expanded blastocyst at 48 h was significantly higher in medium with 1 mM Gln (66.6%; $P < 0.05$) or with 0.125 mM Trp (61.1%; $P < 0.05$). Unexpectedly, Trp increased the percentage of degenerated blastocysts after 48 h (67.7%; $P < 0.05$), while Gln preserved blastocyst viability. These results suggest that Gln may enhance blastocyst hatching, expansion and viability *in vitro*.

Key words: Embryo development; Murine blastocyst; Hatching; Glutamine; Tryptophan

Introduction

Prior to implantation, blastocyst hatching from the zona pellucida (ZP) must occur for the attachment of the blastocyst to the endometrial epithelium and penetration towards the endometrium. Typically, hatching involves blastocyst expansion that causes thinning of the ZP and the synthesis of trypsin-like proteinases by cells of the trophoectoderm. This subsequently induces rupture of the ZP at different sites (1).

The use of amino acids has been the subject of several studies aimed at designing an appropriate culture medium whose composition would improve embryo development *in vitro*. Poor-quality embryos result from suboptimal *in vitro* culture conditions, and under these circumstances only a small proportion of embryos reach the hatched blastocyst stage. Even if culture conditions are sufficient, failure to hatch may be due to the hardening of the ZP and other intrinsic factors (2). For this reason, it was suggested that supplementation of the culture medium with amino acids might improve blastocyst formation. Nevertheless, no studies have been conducted to determine the action of specific amino acids on hatching embryos and the use of L-glutamine (Gln) as a supplement for the culture of embryos is controversial. Rezk et al. (3) demonstrated that Gln increases the number of embryos that reach the blastocyst stage. However, according to Devreker and Hardy (4), addition of Gln to the culture decreases the number of cells of the trophoectoderm and the inner cell mass (ICM). Other studies have reported that Gln supplementation in the culture medium has no effect (5,6). Although this controversy remains, we hypothesized that Gln may improve blastocyst hatching *in vitro*, and we established an artificial model to test whether a single amino acid would affect embryo development.

L-tryptophan (Trp) is emerging as an important amino acid for the development of the blastocyst. The absence of Trp in the medium for embryos grown *in vitro* had a significant impact on processes such as hatching, adhesion to a collagen matrix and embryo outgrowth (7). Tryptophan is a large heterocyclic amino acid essential for metabolism, as well as a substrate for protein synthesis and a precursor of niacin, kynurenine, and quinolinic acid (8).

Currently, there is insufficient evidence regarding the effect of Gln and Trp on blastocyst hatching. For this reason, we carried out a detailed examination of the effect of these two amino acids on blastocyst hatching, expansion and viability. Specifically, the aim of this study was to test the effect of Trp and Gln on murine blastocyst hatching *in vitro*.

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Material and Methods

Reagents

M2 medium, hemin, mineral oil, Trp, and Gln were purchased from Sigma (USA), HAM F12 medium was a product of GIBCO-Invitrogen (USA), and equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) from Intervet (Netherlands).

Animals

Virgin 6- to 8-week-old female BALB/c mice (N = 30) were housed in a standard animal care facility in rooms with a controlled light (12 h light:12 h dark) and controlled temperature (22° to 24°C). Food and water were available *ad libitum*. Superovulation was induced by intraperitoneal injection of 5 IU eCG, followed by 5 IU hCG 48 h later. The females were then bred overnight with BALB/c males (N = 10) of proven fertility and checked for the presence of vaginal plugs the next morning. Mating was assumed to have occurred at midnight, which was taken as time zero. The current study was approved by the Animal Research Committee of the University of Antioquia (approval No. 18, January 28, 2005), Medellín, Colombia, and conducted according to the Guidelines for the Care and Use of Laboratory Animals of the University of Antioquia.

Blastocyst collection and culture

Mice were sacrificed using a CO_2 chamber and the uterine cornua were immediately isolated (one every 5 min) 3.5 days post-coitus (96-98 h after hCG injection). The uterine cornua were handled under the dissecting microscope with surgical tweezers. A 12-inch needle with a 1-mL syringe was introduced into the lumen of the uterus and blastocysts inside the uterine cornua were flushed out into Petri dishes (Falcon, USA) with 3 mL HAM F12 medium. The Petri dishes were incubated for 30 min to allow the blastocysts to sediment. The blastocysts were then washed twice in 20-µL droplets of pre-warmed M2 medium. The embryos were cultured in groups of 3 embryos per well in 96-well dishes (Nunc, USA) in 150 µL M2 medium and overlaid with 50 µL mineral oil. Embryos were stimulated either with M2 medium containing 0.125, 0.25, or 0.5 mM Trp plus 0.2% hemin, or M2 medium supplemented with 1 mM Gln, or M2 medium alone as control. These concentrations of Trp and Gln are optimal for *in vitro* culture of mouse embryos (3,9). The effect of Gln and Trp on embryo development was compared to that of M2 medium alone. Each experiment was performed in triplicate and observations were made every 24 h for 72 h. The blastocysts (N = 262) were divided as follows: M2 alone (N = 60), 0.125 mM Trp (N = 27), 0.25 mM Trp (N = 96), 0.5 mM Trp (N = 28), and 1 mM Gln (N = 51), respectively.

Assessment of blastocyst morphology

Embryo morphology was determined after 24, 48, and 72 h of culture using an inverted phase-contrast microscope (magnification: 10 and 40X). Embryo morphology was classified according to the following criteria: *Blastocyst* (Figure 1A): embryos with an intact ZP, a flat trophoectoderm, a protuberant ICM, and an expanded blastocele; *hatched blastocyst* (Figure 1B): embryos that protruded partially or

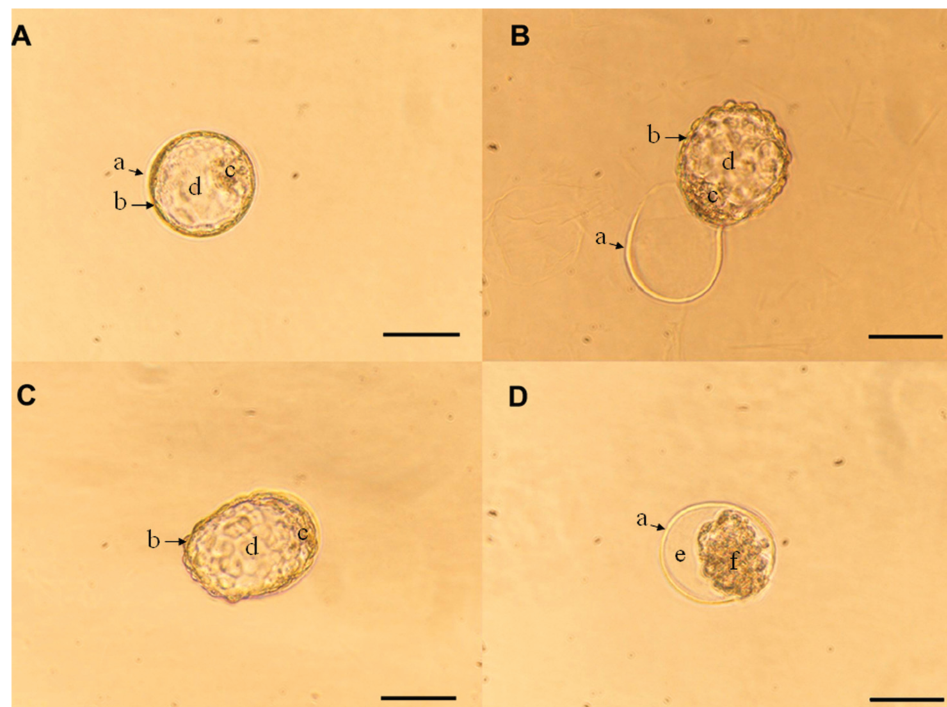

Figure 1. Murine blastocyst development. *A*, *Blastocyst*: embryos with an intact zona pellucida (ZP; a), a flat trophoectoderm (b), a protuberant inner cell mass (c), and an expanded blastocele (d). *B*, *Hatched blastocyst*: embryos that protruded partially or completely from the ZP. *C*, *Expanded blastocyst*: hatched blastocysts with an increase in diameter compared to the blastocyst or hatched blastocyst stages. *D*, *Degenerated blastocyst*: either unexpanded embryos with a space (e) between the trophoectoderm and the ZP, or embryos with cell fragmentation (f). Magnification: 10X. Scale bars = 10 µm.

completely from the ZP; *expanded blastocyst* (Figure 1C): hatched blastocysts with an increase in diameter compared to the blastocyst or hatched blastocyst stages; *degenerated blastocyst* (Figure 1D): either unexpanded embryos with a space between the trophoectoderm and the ZP, or embryos with cell fragmentation.

Statistical analysis

The experiments were performed three times in triplicate, and the results are reported as the mean ± SEM of each experiment. One-way ANOVA with the Tukey multiple comparison post-test was used to compare the different stimuli. P ≤ 0.05 was considered to be significant.

Results

Hatched blastocysts

The percentage of hatching at 24 h in the group of blastocysts stimulated with Gln (100%) was significantly higher than M2 medium alone (21.1 ± 8.4%), 0.25 mM Trp (36.7 ± 10.7%) or 0.5 mM Trp (22.2 ± 11.1%; P < 0.05; Figure 2A). In the group of embryos stimulated with 0.125 or 0.25 mM Trp, the percentage of hatching was higher than in M2 alone (Figure 2A). At 48 h of culture, the percentage of hatching in the groups stimulated with Trp was higher compared to the hatching at 24 h (Figure 2B). The blastocysts maintained without amino acids or with 0.25 mM Trp gradually reached their highest percentage of hatching at 72 h (Figure 2C), whereas blastocysts cultured with Gln reached their highest percentage of hatching at 24 h (Figure 2A). After 48 h, blastocyst hatching was lower in the groups cultured with 0.125 and 0.5 mM Trp as compared to the result observed in the groups of blastocysts maintained in M2 alone or supplemented with 0.25 mM Trp (Figure 2C).

Expanded blastocysts

Blastocyst expansion was observed after 48 h of culture and was significantly higher in the group of blastocysts cultured with Gln (66.6 ± 0.2%), compared to either M2 alone (9.7 ± 7.9%), 0.25 mM Trp (19.1 ± 6.1%) or 0.5 mM Trp (7 ± 3.5%; P < 0.05; Figure 3A). Moreover, the percentage of expanded blastocysts was significantly higher in the presence of 0.125 mM Trp (61.1 ± 16.0%) compared to M2 alone or to 0.5 mM Trp (P < 0.05; Figure 3A), and was similar to the group stimulated with Gln (Figure 3A). After 72 h of culture, the expansion of the blastocysts increased to 96.2 ± 2.1% in the group of blastocysts stimulated with Gln compared to expansion at 48 h (Figure 3B). No statistically significant differences were observed in the groups stimulated with Trp compared to M2 alone (Figure 3B). These results show that treatment with Gln or 0.125 mM Trp after hatching could be useful to increase the rate of expanded blastocysts.

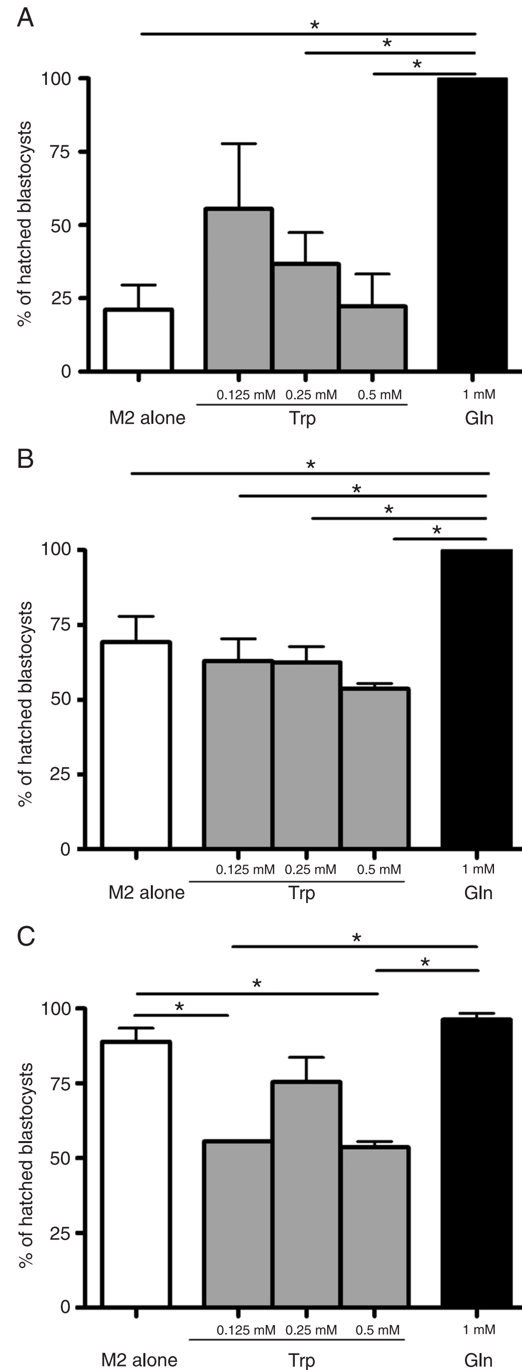

Figure 2. Induction of blastocyst hatching by Gln. The figure shows the percentage (means ± SEM) of hatched blastocysts at 24, 48, and 72 h of incubation in M2 medium alone (open bar), or medium plus 0.125 mM Trp, 0.25 mM Trp, 0.5 mM Trp (gray bars), or 1 mM Gln (black bar). Hatching of the blastocysts cultured in M2 medium containing 1 mM Gln was significantly higher than in the other conditions at 24 (A) and 48 h (B). Embryo hatching did not increase in the groups stimulated with 0.125 and 0.5 mM Trp at 72 h (C) compared to 48 h. *P < 0.05 (one-way ANOVA).

Degenerated blastocysts

The percentage of degenerated blastocysts was determined after 48 h of culture. The lowest percentage of degenerated blastocysts was observed in groups cultured with Gln (0%), M2 alone (2.7 ± 2.7%) and 0.25 mM Trp (6.1 ± 1.4%; Figure 4A). Unexpectedly, the highest percentage of degenerated blastocysts was obtained in groups of blastocysts stimulated with 0.5 or 0.125 mM Trp compared to M2 alone or Gln (Figure 4A). After 72 h of culture, the percentage of degenerated blastocysts increased to 16.1 ± 6.2% with 0.25 mM Trp, to 67.7 ± 6.5% with 0.5 mM Trp and it increased drastically in the group stimulated with 0.125 mM Trp, reaching 48.1 ± 19.5% (Figure 4B). After 96 h of culture the percentage increased in all groups except the group with Gln (data not shown). Overall, blastocyst viability was preserved until 72 h in the groups cultured with M2 alone, 0.25 mM Trp, or Gln (Figure 4). On the other hand, 0.125 and 0.5 mM Trp had a detrimental effect on embryo viability beyond 48 h of culture.

Discussion

In addition to rupture of the ZP by the expanding blastocyst or proteases, blastocyst hatching also requires dynamic changes in the cytoskeleton (1). These cytoskeletal changes occur during hatching because the trophoectoderm

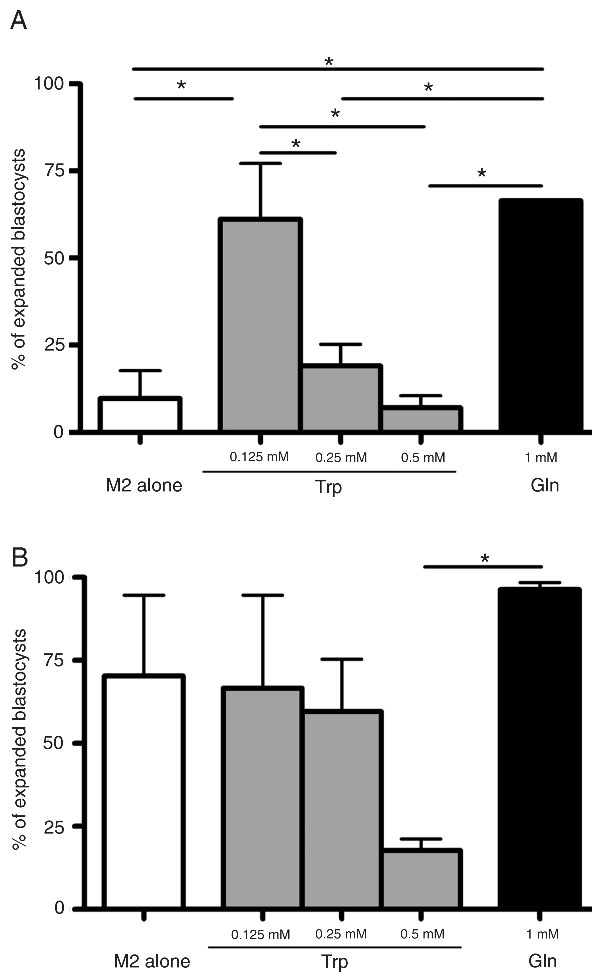

Figure 3. Effect of Trp and Gln on blastocyst expansion. The figure shows the percentage (means ± SEM) of expanded blastocysts at 48 and 72 h of incubation in M2 medium alone (open bar), or medium plus 0.125 mM Trp, 0.25 mM Trp, 0.5 mM Trp (gray bars), or 1 mM Gln (black bar). The expansion of the blastocysts cultured in the medium containing 1 mM Gln or 0.125 mM Trp was significantly higher than in the other conditions at 48 h (A); expansion of the blastocysts at 72 h was highest in the medium containing 1 mM Gln (B). *P < 0.05 (one-way ANOVA).

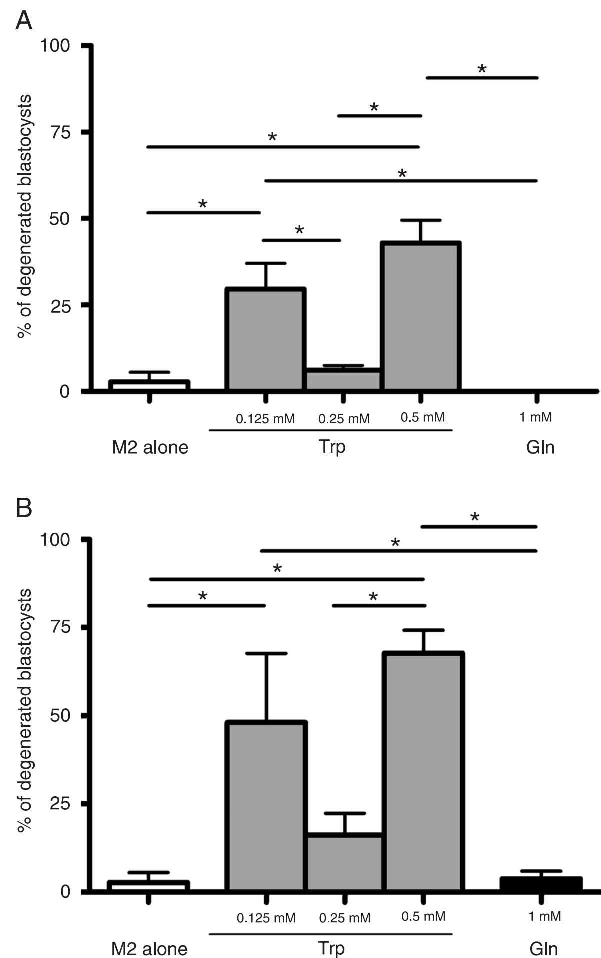

Figure 4. Blastocyst viability is preserved by Gln. The figure shows the percentage (means ± SEM) of degenerated blastocysts at 48 and 72 h of incubation in M2 medium alone (open bar), or 0.125 mM Trp, 0.25 mM Trp, 0.5 mM Trp (gray bars), or 1 mM Gln (black bar). The degeneration of blastocysts cultured in M2 medium containing 1 mM Gln or M2 alone was significantly lower than in the other conditions at 48 (A) and 72 h (B). *P < 0.05 (one-way ANOVA).

protrudes through a narrow hole in the ZP, which allows the entire blastocyst to escape from the ZP. This process may imply complex signaling mechanisms orchestrated by amino acids, growth factors and cytokines. In this study, we show that Gln may positively modulate blastocyst protrusion from the ZP, inducing 100% hatching after 24 h. Gln is involved in different cell signaling pathways, suggesting that it has the potential to determine distinct cell fates, including growth, differentiation and death (10).

In order to characterize other possible actions of Gln and Trp, we determined blastocyst viability and observed that Gln, but not Trp, prevented blastocyst degeneration at all times; whereas Trp had a detrimental effect at 48 h that was maintained until 72 h of culture. This result is consistent with previous studies showing the influence of Gln on mouse embryo development *in vitro* (11,12). For this reason, we propose that Gln has a protective effect on embryo development *in vitro*.

When the embryos were cultured in M2 medium with Trp, we observed an increase in the percentage of degenerated blastocysts at 72 h. However, we could not explain why 0.25 mM Trp did not have a defined detrimental effect as was observed with 0.125 and 0.5 mM Trp. Although the experiments were performed three times in triplicate, we always observed the same pattern of viability with 0.25 mM Trp. Moreover, the low dispersion of the data did not indicate variations between experiments. Further experiments are required to explain why 0.25 mM Trp had this unexpected effect.

Initially, we hypothesized that Trp would play an important role during embryo development. Indeed, 0.125 mM Trp promoted blastocyst expansion after 48 h. Van Winkle et al. (13) have described in detail the $B^{0,+}$ system, an amino acid transporter in murine embryos that regulates the entry of Trp, leucine, isoleucine, and phenylalanine into the blastocysts. It is clear from our results that 0.125 mM Trp promotes blastocyst expansion during the first 48 h, whereas higher Trp concentrations have no effect. This fundamental action of Trp at low concentrations might reflect certain requirements of the embryo, such as its expansion under physiological conditions *in vivo* during the preimplantation period. It is possible that such a phenomenon occurs, based on the findings of Harris et al. (14) and Hugentobler et al. (15) who observed that Trp, methionine and asparagine were present at low concentrations in murine (14) and bovine (15) reproductive tracts compared to other amino acids. Thus, we conclude that 0.125 mM Trp has a positive effect on the percentage of expanded blastocysts just after hatching, but a detrimental effect prior to hatching.

We showed in this study that Gln induces blastocyst hatching and preserves embryo viability. Furthermore, both Gln and 0.125 mM Trp regulate blastocyst expansion. Therefore, we suggest that supplementation of the culture medium with Gln is useful to improve embryo development *in vitro*.

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References

1. Niimura S, Fujii M. A morphological study of blastocyst hatching in the mouse and rat. *J Reprod Dev* 1997; 43: 295-302.
2. De Vos A, Van Steirteghem A. Zona hardening, zona drilling and assisted hatching: new achievements in assisted reproduction. *Cells Tissues Organs* 2000; 166: 220-227.
3. Rezk Y, Huff C, Rizk B. Effect of glutamine on preimplantation mouse embryo development i*n vitro*. *Am J Obstet Gynecol* 2004; 190: 1450-1454.
4. Devreker F, Hardy K. Effects of glutamine and taurine on preimplantation development and cleavage of mouse embryos *in vitro*. *Biol Reprod* 1997; 57: 921-928.
5. Du ZF, Wales RG. Some effects of genotype and composition of the culture medium on the development of mouse zygotes *in vitro*. *Reprod Fertil Dev* 1993; 5: 405-415.
6. Summers MC, Biggers JD. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. *Hum Reprod Update* 2003; 9: 557-582.
7. Spindle AI, Pedersen RA. Hatching, attachment, and outgrowth of mouse blastocysts *in vitro*: fixed nitrogen requirements. *J Exp Zool* 1973; 186: 305-318.
8. Tatsumi K, Higuchi T, Fujiwara H, Nakayama T, Egawa H, Itoh K, et al. Induction of tryptophan 2,3-dioxygenase in the mouse endometrium during implantation. *Biochem Biophys Res Commun* 2000; 274: 166-170.
9. Lane M, Hooper K, Gardner DK. Effect of essential amino acids on mouse embryo viability and ammonium production. *J Assist Reprod Genet* 2001; 18: 519-525.
10. Newsholme P, Procopio J, Lima MM, Pithon-Curi TC, Curi R. Glutamine and glutamate - their central role in cell metabolism and function. *Cell Biochem Funct* 2003; 21: 1-9.
11. Biggers JD, McGinnis LK, Lawitts JA. Enhanced effect of glycyl-L-glutamine on mouse preimplantation embryos *in vitro*. *Reprod Biomed Online* 2004; 9: 59-69.
12. Summers MC, McGinnis LK, Lawitts JA, Biggers JD. Mouse embryo development following IVF in media containing either L-glutamine or glycyl-L-glutamine. *Hum Reprod* 2005; 20: 1364-1371.
13. Van Winkle LJ, Tesch JK, Shah A, Campione AL. System

$B^{0,+}$ amino acid transport regulates the penetration stage of blastocyst implantation with possible long-term developmental consequences through adulthood. *Hum Reprod Update* 2006; 12: 145-157.
14. Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology* 2005; 64: 992-1006.
15. Hugentobler SA, Diskin MG, Leese HJ, Humpherson PG, Watson T, Sreenan JM, et al. Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. *Mol Reprod Dev* 2007; 74: 445-454.