Original Article

Mediating Trophoblast Uptake of Methylaminoisobutyric Acid and Glutamine by Absence of Extra- or Intracellular Glutamine

Boonrit Thongsong*

Abstract

Glutamine is known to modulate the uptake activity of nutrient transporters in mammalian cells. The aim of the study was to elucidate the influence of absence of extra- or intracellular glutamine on the uptake activity of the amino acid transport system A in placental BeWo cells. Extracellular glutamine was absent by culturing the cells in regular medium without glutamine. Intracellular glutamine was depleted by culturing the cells in regular medium without glutamine and by treating the cells for 16 hours with methionine sulfoximine (MSX), an inhibitor of glutamine synthetase. When BeWo cells were treated with glutamine-free culture medium for 1-30 hours, the uptakes of MeAIB and glutamine were higher than those with regular medium added glutamine. In addition, when these cells were treated with the absence of extracellular glutamine with or without 2 mM MSX, the uptake of not only methylaminoisobutyric acid (MeAIB) but also glutamine was measured in the presence of increasing concentrations (0-10 mM) of glutamine and of MeAIB, respectively. The data show that the uptake of MeAIB was inhibited by MSX treatment and the entry of MeAIB into the cells was effectively blocked by glutamine. In contrast, the uptake of glutamine was not affected by MSX treatment and that entry of glutamine into the cells was not affected by MeAIB. The reason why glutamine uptake in the placental cells was not affected by MSX treatment is there are some other amino acid transport systems for entry of glutamine instead of system A. The elucidated role of such regulation of glutamine should aid the rational utility of this amino acid in acting nutrient.

Keywords: amino acid transport system A, methionine sulfoximine, methylaminoisobutyric acid, glutamine, trophoblast uptake

Department of Animal Husbandry, Faculty of Veterinary Science, Chulalongkorn University Bangkok, 10330, Thailand
***Corresponding author: E-mail:** Boonrit.T@chula.ac.th*

บทคัดย่อ

เหนี่ยวนำการนำเข้าเมทธิลอะมิโนไอโซบิวทีริคแอซิดและกลูทามีนสู่เซลล์รกในสภาวะที่ไม่มีกลูทามีนภายนอกหรือภายในเซลล์

*บุญฤทธิ์ ทองทรง**

เป็นที่ทราบกันว่ากรดอะมิโนกลูทามีน สามารถควบคุมการทำงานของตัวขนส่งสารอาหารในเซลล์ของสัตว์เลี้ยงลูกด้วยนม วัตถุประสงค์การศึกษาครั้งนี้เพื่อเข้าใจอิทธิพลของสภาวะที่ไม่มีกรดอะมิโนกลูทามีน ภายนอกหรือภายในเซลล์รกที่เพาะเลี้ยงต่อการทำหน้าที่นำเข้ากรดอะมิโนของตัวขนส่งระบบเอ วิธีการทำให้ไม่มีกลูทามีนภายนอกเซลล์คือใช้อาหารเลี้ยงเซลล์ตามปกติแต่ไม่มีกรดอะมิโนกลูทามีน ส่วนวิธีการทำให้กลูทามีนลดลงภายในเซลล์คือ ใช้อาหารเลี้ยงเซลล์ตามปกติที่ไม่มีกรดอะมิโนกลูทามีน และเติมเมทไธโอนีนซัลโฟซิมีน ซึ่งทำหน้าที่ยับยั้งเอนไซม์กลูทามีนซินทีเทส เป็นระยะเวลานาน 16 ชั่วโมง ทำการศึกษาการนำเข้าเมทธิลอะมิโนไอโซบิวทีริคแอซิดและกลูทามีนผ่านเซลล์ดังกล่าว โดยเมื่อใช้อาหารเลี้ยงเซลล์ตามปกติแต่ไม่มีกรดอะมิโนกลูทามีนเป็นระยะเวลาตั้งแต่ 1 ถึง 30 ชั่วโมงพบว่า กรดอะมิโนทั้งสองตัวผ่านเข้าสู่เซลล์ได้มากกว่าเมื่อเปรียบเทียบกับการใช้อาหารเลี้ยงเซลล์ที่เติมกลูทามีนตามปกติ นอกจากนี้เมื่อทำการศึกษาการนำเข้าเมทธิลอะมิโนไอโซบิวทีริคแอซิดและกลูทามีนผ่านเซลล์ดังกล่าว โดยใช้กลูทามีนและเมทธิลอะมิโนไอโซบิวทีริคแอซิดเป็นสารตั้งต้นตามลำดับที่ระดับความเข้มข้นเพิ่มขึ้นจาก 0 ถึง 10 นาโนโมลาร์ ในสภาวะที่ไม่มีกลูทามีนภายนอกเซลล์และได้รับหรือไม่ได้รับเมทไธโอนีนซัลโฟซิมีน ข้อมูลผลการศึกษาแสดงให้ทราบว่า การนำเข้าของเมทธิลอะมิโนไอโซบิวทีริคแอซิด ถูกยับยั้งโดยเป็นผลมาจากการให้เมทไธโอนีนซัลโฟซิมีน และการผ่านเข้าสู่ภายในเซลล์ของเมทธิลอะมิโนไอโซบิวทีริคแอซิดถูกยับยั้งโดยกลูทามีน ในทางตรงกันข้าม การนำเข้าของ กลูทามีนไม่ได้รับผลมาจากการให้เมทไธโอนีนซัลโฟซิมีน และการผ่านเข้าสู่ภายในเซลล์ของกลูทามีนไม่เป็นผลโดยการใช้เมทธิลอะมิโนไอโซบิวทีริคแอซิดเป็นสารตั้งต้น เหตุผลว่าทำไมการนำเข้าของกลูทามีนไม่ได้เป็นผลมาจากการได้รับเมทไธโอนีนซัลโฟซิมีนนั้น เนื่องมาจากมีตัวขนส่งกรดอะมิโนระบบอื่นๆที่นอกเหนือ จากระบบเอ สามารถนำกลูทามีนผ่านเข้าเซลล์รกได้ ฉะนั้นการเข้าใจบทบาทของการควบคุมกรดอะมิโนกลูทามีนช่วยเพิ่มเหตุผลในการใช้งานกรดอะมิโนดังกล่าวอย่างเหมาะสมแก่การเป็นสารอาหารประการหนึ่ง

คำสำคัญ: ขนส่งกรดอะมิโนระบบเอ เมทไธโอนีนซัลโฟซิมีน เมทธิลอะมิโนไอโซบิวทีริคแอซิด กลูทามีน การนำเข้าเซลล์รก

ภาควิชาสัตวบาล คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ
***ผู้รับผิดชอบบทความ E-mail:** Boonrit.T@chula.ac.th*

Introduction

The most abundant amino acid in bloodstream is glutamine. It is an important molecule for physiologic requirements such as a protein synthesis and a variety of additional biological function. Furthermore, some pathological conditions such as septic shock were found in glutamine starvation (Karinch et al., 2001). Intracellular concentration of glutamine is very high when it plays a central role as a ready source of nitrogen, carbon, and energy in various metabolic processes such as to influence the cellular levels of glutathione (GSH) (Oehler and Roth. 2003). Changes in GSH status of the cells and the resultant redox state of the cell might affect amino acid transport system A activity. The system A using methylaminoisobutyric acid (MeAIB) transcellular function which accepts glutamine and other short-chain neutral amino acids as substrates (Ganapathy et al., 2003) is regulated by many effectors. This amino acid transport system is one of the transporters that mediates glutamine entry into normal placental syncytiotrophoblast at the brush border membrane (Novak and Beveridge, 1997). Accumulating evidence has shown that glutamine is a key acting nutrient. In fetal-placental nutrient exchange, glutamine supports tissue homeostasis by participating in intercellular substrate cycles. Since the fetus needs a large amount of glutamine from the early period of gestation, systemic glutamine deficiency can lead to neonatal multiple organ failure (Neu. 2001). Additionally, glutamine appears essential for a viability and growth of cultured cells. Mammalian cells take up glutamine from extracellular medium by several active and passive amino acid transports (Ganapathy et al., 2003). In fact, removing extracellular glutamine will not affect the endogenous synthesis of glutamine because intracellular glutamine is also synthesized by the amidation of

glutamate. This ATP-dependent reaction is catalyzed by glutamine synthetase, an enzyme inhibitable by the glutamine analog methionine sulfoximine (MSX). Thongsong (2012[b]) demonstrated that treatment with 2 mM MSX had no effect on system A activity when trophoblast cells were treated in the presence of glutamine. However, when cultured in the absence of glutamine, treatment with MSX reduced system A activity. The effect of MSX on inhibition of system A activity was increased as the concentration of MSX increased.

It is well established that glutamine is the preferred substrate for mammalian cells, including placental cells. Thus, it is important for the normal growth and maturation of placenta. Serving as a barrier between the mother and the fetus, placental cells mediate the uptake into and release of amino acids from the cells by a variety of amino acid transporters for fetal growth and development (Ganapathy et al., 2003). These transporters are, thus, vital for the homeostasis of amino acids in cells and some of these are capable of transporting glutamine. A few studies had investigated the effects of extra- and intratrophoblast glutamine on regulation of system A activity. The aim of the present study was to elucidate the influence of extracellular glutamine absence or/and intracellular glutamine depletion on the uptake activity of the amino acid transport system A in BeWo cells, a human placental choriocarcinoma cell line as a model for trophoblast. A number of amino acid transport systems had been reported in the brush border membrane of these cells, of which system A, namely ATA1 and ATA2, are of special importance (Jones et al., 2002). Birth weight of baby under some conditions had been shown to correlate with system A transport activity in the placental brush border membrane (Glazier et al., 1997; Harrington et al., 1999). Furthermore, the inhibition of system A activity in the trophoblasts by interleukin - 1beta may be subjected to alterations under various physiological and pathological conditions (Thongsong et al., 2005). Therefore, information on the elucidate effects and role of such regulation of glutamine should aid the rational utility of this amino acid in acting nutrient and may be responsible for understanding of placental-fetal growth and development.

Materials and Methods

Cell culture and reagents: The BeWo choriocarcinoma cell line, cell culture media, fetal bovine serum, unlabeled and radiolabeled amino acids of [^{14}C]α-(methylamino) isobutyric acid (MeAIB) (55 mCi/mmol) and L-[^{3}H] glutamine (45 Ci/mmol) were provided by Professor Dr. Vadivel Ganapathy and Professor Dr. Puttur D Prasad.

Cell culture and treatment: BeWo cells were cultured in 12-well culture plates for uptake assays in DMEM/F-12 (50:50) medium containing 2.5 mM glutamine and supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The culture conditions and the rationale of intracellular glutamine depletion for the study were as previously described (Thongsong, 2012[a]). Confluent cultures were treated in a glutamine-free culture medium with and without MSX for 16 hours. In brief, these culture conditions lead to the absence of extracellular glutamine and the depletion of intracellular glutamine because of the lack of glutamine in the medium and also because of the inhibition of endogenous synthesis of glutamine by MSX. Subsequently, the cells were used for the MeAIB and glutamine uptake measurements.

MeAIB and glutamine uptake measurements: Uptake measurements were carried out at 37^{0}C. The medium was aspirated and the cell monolayer was washed once with the uptake buffer. Uptake was then initiated by the addition of 500 µl of uptake buffer containing 0.5 µCi of radiolabeled MeAIB or glutamine. For time course of [^{14}C] α-MeAIB uptake in BeWo cells, the activity of system A was then measured using MeAIB (4 µM) as the substrate. In one set of experiments, the uptake of 5 µM MeAIB was measured after the absence and presence of extracellular glutamine for 1 to 30 hours as well as in the presence of increasing concentrations (0-10 mM) of glutamine. Another set of experiment, the uptake of 5 µM glutamine was measured after the absence and presence of extracellular glutamine for 1 to 30 hours as well as in the presence of increasing concentrations (0-10 mM) of MeAIB. The incubation was continued for a desired length of time, following which the uptake was terminated by aspirating the uptake medium. After the termination of the uptake, the cells were washed two times with 1.5 ml of ice-cold uptake buffer. The cells were then solubilized with 0.5 ml of 1% SDS/0.2 N NaOH and transferred to scintillation vials for the determination of the radioactivity associated with the cells. The composition of the uptake buffer was 25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose.

Statistical analyses: Experiments were made in triplicate and each experiment was repeated three times. The results were given as means±SEM.

Results

Initial time course of [^{14}C] α-MeAIB transport

The study was started by initial time course of MeAIB uptake. It showed that the uptake was linear up to 40 minutes as shown in Fig 1. Therefore, all subsequent measurements were done within this linear phase of uptake. Thus, these experiments were done with 10 and 20 minutes incubations.

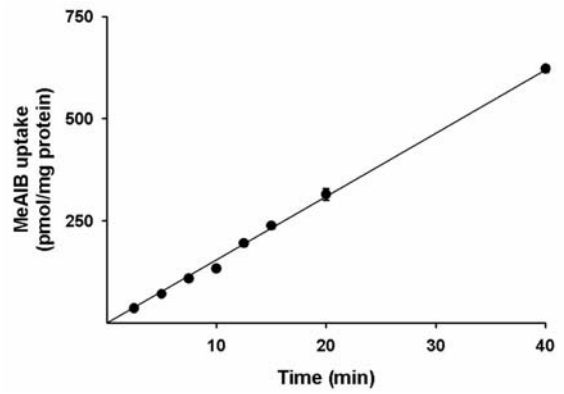

Figure 1 Initial time course of [^{14}C] α-MeAIB transport in BeWo cells was measured at 37^{0}C in the presence of Na^+. The concentration of [^{14}C] α-MeAIB was 10 μM (0.25 μCi/assay). This study shows that the uptake of MeAIB was linear up to 40 minutes.

Time course effect of extracellular glutamine absence on the uptake activity of the amino acid transport system A in BeWo cells

The studies investigated time course effect of extracellular glutamine absence on the uptake activity of MeAIB and glutamine in BeWo cells. These cells were treated in glutamine-free culture medium or in regular medium with glutamine for increasing time during 1-30 hours. The uptake of 5 μM MeAIB and glutamine was measured in the culture medium condition. Surprisingly, the data show that the uptake of MeAIB was significantly higher in treated cells without extracellular glutamine than in those with extracellular glutamine throughout 30 hours (Fig 2). This phenomenon consisted of increasing uptake of glutamine in treated cells with glutamine-free culture medium when compared to those with regular medium added glutamine throughout 30 hours as shown in Fig 3.

Influence of extracellular glutamine absence or intracellular glutamine depletion on the uptake activity of the amino acid transport system A in BeWo cells

The studies investigated the effect of extracellular glutamine absence or intracellular glutamine depletion on the uptake of glutamine and MeAIB by system A transporters in BeWo cells. Confluent cells were treated in the absence of extracellular glutamine with or without MSX for 16 hours. In one set of experiments, uptake of 5 μM MeAIB was measured in the presence of increasing concentrations of glutamine. The data show that the uptake of MeAIB was inhibited by MSX treatment and the entry of MeAIB into the cells was effectively blocked by glutamine as the concentration of glutamine increased (Fig 4).

In the second set of experiments, uptake of 5 μM glutamine was measured in the presence of increasing concentrations of MeAIB. In contrast to the uptake of MeAIB, the data show that the uptake of glutamine was not affected by MSX treatment and

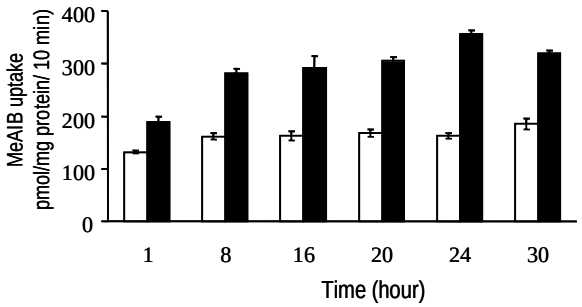

Figure 2 Time course effect of extracellular glutamine absence on MeAIB uptake in BeWo cells. The uptake activity was measured at 37^{0}C in the presence of Na^+. BeWo cells were treated in a glutamine-free culture medium (filled bars) or in regular medium with glutamine (open bars). The uptake of MeAIB was measured after the absence and presence of extracellular glutamine for 1 to 30 hours.

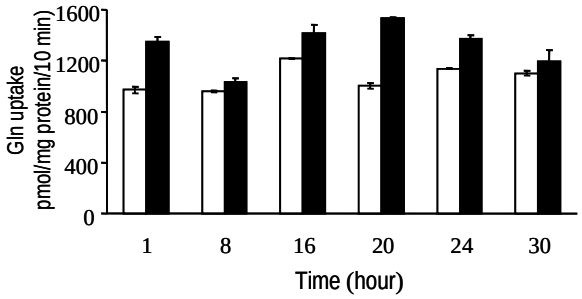

Figure 3 Time course effect of extracellular glutamine absence on glutamine uptake in BeWo cells. The uptake activity was measured at 37^{0}C in the presence of Na^+. BeWo cells were treated in a glutamine-free culture medium (filled bars) or in regular medium with glutamine (open bars). The uptake of glutamine was measured after the absence and presence of extracellular glutamine for 1 to 30 hours.

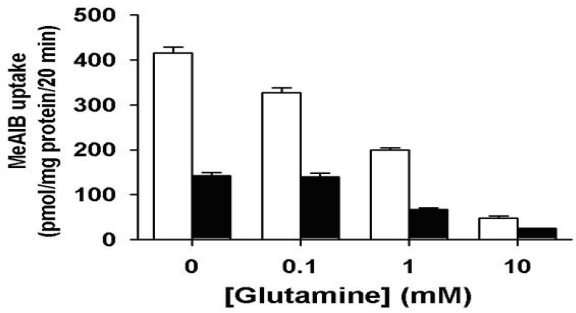

Figure 4 Role of amino acid transport system A in the cellular uptake of MeAIB. BeWo cells were treated in the absence of extracellular glutamine with (filled bars) or without (open bars) MSX (2 mM) for 16 hours. The uptake of 5 μM MeAIB was measured in the presence of increasing concentrations (0-10 mM) of glutamine.

that entry of glutamine into the cells was not affected by MeAIB (Fig 5). Surprisingly, the uptake of glutamine is slightly higher in MSX-treated cells compared to the glutamine uptake in non-MSX-treated cells.

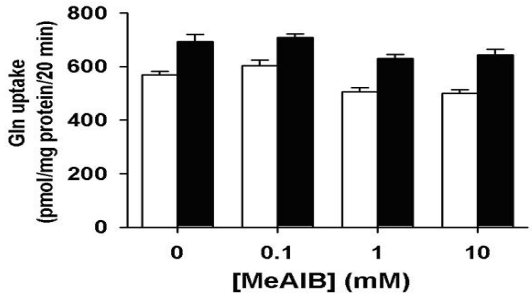

Figure 5 Role of amino acid transport system A in the cellular uptake of glutamine. BeWo cells were treated in the absence of extracellular glutamine with (filled bars) or without (open bars) MSX (2 mM) for 16 hours. The uptake of 5 µM glutamine was measured in the presence of increasing concentrations (0-10 mM) of MeAIB.

Discussion

As for *in vitro* model for studying trophoblast transcellular transport, BeWo cell line was well recognized in one out of two trophoblast cell lines that had been shown to form confluent monolayer and successfully utilized to investigate the asymmetric transcellular transport of multiple nutrients and compounds (Bode et al., 2006). Furthermore, this cell line had been shown to be a valid model for studying the transplacental transport of nanoparticles (Cartwright et al., 2012). Transplacental amino acid transport is vital for the development, growth and function of not only the placenta but fetus as well. This study described the influence of extracellular glutamine absence and/or intracellular glutamine depletion on the uptake activity of amino acid transport system A in placental BeWo cells. It had been reported that system A played a key role in glutamine transport from maternal side into placental cells (Novak and Beveridge, 1997). There are two routes to supply glutamine to fetus; uptake from maternal circulation and production in the placenta (Neu, 2001). Thus, extracellular and intracellular glutamine status in these cells might modulate the system A activity. In the consistent study of Thongsong (2012[b]), removal of extracellular glutamine without affecting the endogenous synthesis of glutamine stimulated the activity of system A. But, removal of extracellular glutamine and simultaneous inhibition of endogenous synthesis of glutamine decreased the activity of system A. Subsequently, kinetic studies defining the kinetic parameters of the system A activity suggested that treatment with MSX most likely resulted in a decrease in the surface density of functional system A proteins in the plasma membrane of the cells. Under condition of extracellular glutamine absence and simultaneous inhibition of endogenous synthesis of glutamine, the uptake of glutamate, alanine, glycine, taurine and carnitine was reduced to a varied extent while the uptake of serine, threonine and histidine was not influenced. Thus, intracellular glutamine was obligatory for maintenance of optimal activity of amino acid uptake in the placental cells (Thongsong, 2012[a]).

Preliminary evidence suggests that placental functions of specific transport proteins are influenced by extracellular concentrations of nutrients. Dall'Asta et al. (1990) reported that system A could mediate glutamine uptake, but its contribution became appreciable only after prolonged periods of amino acid starvation. Interestingly, the uptake activity of MeAIB and glutamine as substrate of system A transporter was higher in treated cells without extracellular glutamine than in those with extracellular glutamine throughout 30 hours (Fig 2 & 3). Thus, as long as extracellular glutamine was absent, increases in MeAIB and glutamine uptake were adaptive regulation or adaptive increase might be considered an attempt to recover or sufficiently supply cells after induced by without extracellular glutamine. These data show that placental cells adapted in vitro to glutamine availability and resulted in increasing activity of system A in treated cells without extracellular glutamine. To explain the increasing system A activity, Jone et al. (2006) demonstrated that the amount of MeAIB transferred through the paracellular routes was lower than 10% total. Therefore, response to restricted amino acid availability was due to changes in specific transport mechanism rather than a generalized change in the physical properties of the cell layer. Additionally, it was intriguing that, in spite of the activity of system A being down regulated in MSX-treated cells, the uptake of glutamine was higher in MSX-treated cells than in absence of MSX and glutamine. The data obtained not only confirm the results from my previous report (Thongsong, 2012[a]) that the uptake of glutamine was higher in MSX-treated cells, but also show in this study that the glutamine uptake into BeWo cells was not affected by the presence of extracellular MeAIB (Fig 5). In contrast, the uptake of MeAIB was decreased by MSX treatment and the entry of MeAIB into the cells was effectively blocked by glutamine (Fig 4). Only very little glutamine in the extracellular medium was needed to block the influence of MSX on system A activity. The concentration of glutamine that caused 50% blockade of the MSX-induced system A inhibition was 49$\pm$6 µM. (Thongsong, 2012b). Thus, though substrate of system A was MeAIB and glutamine, Other amino acid transport systems could uptake the observed entry of glutamine into the BeWo cells. The activity of these non-system A transporters mediating the uptake of glutamine was stimulated in cells treated with MSX and, therefore, glutamine uptake was higher in MSX treated cells. The effect of glutamine depletion on amino acid transport was not limited to specific amino acid. Thus, extracellular and intracellular glutamine appeared to have a differential effect on the regulation of some amino acid activities (Thongsong, 2012[a]). An adequate and sustained elevation of tissue glutamine level was essential for correcting glutamine deficiency and overcoming the threshold for eliciting benefits of exogenous glutamine (Xue et al., 2011).

It is possible to conclude from these studies and the previous reports that amino acid transport system A accepted MeAIB as well as glutamine as substrates. The extracellular glutamine limitation mediated in the response to activity of system A and

showed placental cells in vitro to glutamine availability by adaptive increased activity of system A. Furthermore, amino acid transport systems other than system A were primarily responsible for the observed entry of glutamine into the BeWo cells. This is the reason why glutamine uptake was not affected by MSX treatment. These studies now provide the means to further delineating its elucidated effect and role of such regulation on extra- or/and intracellular glutamine that should aid the rational utility of this amino acid in acting nutrient.

Acknowledgments

The author would like to thank Professor Dr. Vadivel Ganapathy and Professor Dr. Puttur D Prasad from Georgia's Health Sciences University, USA, for their kindness and significant advice.

References

Bode CJ, Jin H, Rytting E, Silverstein PS, Young AM and Audus KL 2006. *In vitro* models for studying trophoblast transcellular transport. Methods Mol Med. 122: 225-239.

Cartwright L, Poulsen MS, Nielsen HM, Pojana G, Knudsen LE, Saunders M and Rytting E 2012. *In vitro* placental model optimization for nanoparticle transport studies. Int J Nanopar. 7: 497-510.

Dall'AstaV, Rossi PA, Bussolato O, Guidotti GG and Gazzola GC 1990. The transport of L-glutamine into cultured human fibroblasts. Biochim Biophys Acta. 1052: 106-112.

Ganapathy V, Inoue K, Prasad PD and Ganapathy ME 2003. Cellular uptake of amino acids: systems and regulation. In: LA Cynober (ed), Metabolic and therapeutic aspects of amino acids in clinical nutrition, CRC Press, NY: 63-78.

Glazier JD, Cetin I, Perugino G, Ronzoni S, Grey AM, Mahendran D, Marconi AM, Pardi G and Sibley CP 1997. Association between the activity of the system A amino acid transporter in the microvillous plasma membrane of the human placenta and severity of fetal compromise in intrauterine growth restriction. Pediatr Res. 42: 514-519.

Harrington B, Glazier J, D'Souza S and Sibley C 1999. System A amino acid transporter activity in human placental microvillous membrane vesicles in relation to various anthropometric measurements in appropriate and small for gestational age babies. Pediatr Res. 45: 810-814.

Jones CR, Srinivas SR, Devoe LD, Ganapathy V and Prasad PD 2002. Inhibition of system A amino acid transport activity by ethanol in BeWo choriocarcinoma cells. Am J Obstet Gynecol. 187: 209-216.

Jones HN, Ashworth CJ, Page KR and McArdle HJ 2006. Expression and adaptive regulation of amino acid transport system A in a placental cell line under amino acid restriction. Reprod. 131: 951-960.

Karinch AM, Pan M, Lin CM, Strange R and Souba WW 2001. Glutamine metabolism in sepsis and infection. J Nutr. 131: 2535S-2538S; discussion 2550S-2551S.

Neu, J. 2001. Glutamine in the fetus and critically ill low birth weight neonate; metabolism and mechanism of action. J Nutr.131 : 2585S-2589S, discussion 2590S.

Novak DA and Beveridge MJ 1997. Glutamine transport in human and rat placenta. Placenta. 18: 379-386.

Oehler R and Roth E 2003. Regulative capacity of glutamine. Curr Opin Clin Nutr Metab Care. 6: 277-282.

Thongsong B 2012[a]. The depletion of intracellular glutamine by methionine sulfoximine on amino acid uptake in placental cells (BeWo). Thai J Vet Med. 42(2): 209-212.

Thongsong B 2012[b]. Influence of intracellular glutamine depletion on regulation of amino acid transport system A in placental (BeWo) cells. Thai J Vet Med. 42(3): 267-273.

Thongsong B, Subramanian RK, Ganapathy V and Prasad PD 2005. Inhibition of amino acid transport system A by interleukin-1beta in trophoblasts. J Soc Gynecol Invest. 12: 495-503.

Xue H, Sufit AJD and Wischmeyer PE 2011. Glutamine therapy improves outcome of *in vitro* and *in vivo* experimental colitis models. J Parenter Enteral Nutr. 35(2): 188-197.