Short communication: Glutamine increases autophagy of liver cells in weaned calves

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ABSTRACT

The objectives of this study were to determine the effects of an increased jugular supply of L-Gln on the autophagy of weaned calves. At 35 d of age, 24 Holstein calves (initial body weight of 50 ± 0.5 kg; 35 ± 2 d of age) were randomly allocated to 4 treatments, with each treatment including 5 male calves and 1 female calf. Holstein calves were assigned to treatments of (1) intravenous infusion of 2 L of 0.85% NaCl (control group) (2) intravenous infusion of 8 g/d of L-Gln mixed with 2 L of 0.85% NaCl solution, (3) intravenous infusion of 16 g/d of L-Gln mixed with 2 L of 0.85% NaCl solution, and (4) intravenous infusion of 32 g/d of L-Gln mixed with 2 L of 0.85% NaCl. The infusion was administered 2 h/d for 7 consecutive days starting on d 1 after weaning. Feed and water were freely available to all calves. All calves were killed on d 7 postweaning to measure the autophagy of liver cells. The level of autophagy in liver cells was improved when the Gln infusion dose increased.

Key words: calf, glutamine, autophagy

Short Communication

Weaning, the transition from the ingestion of maternal milk to solid foods, is a particularly vulnerable period for mammals, with increased incidences of malnutrition, intestinal infections, and poor growth (Blecha et al., 1983; Wilson et al., 1989; Spreeuwenberg et al., 2001). During periods of physiological stress, such as trauma, burns, and postoperative recovery, supplemental Gln has proved beneficial (Lacey and Wilmore, 1990). Some of the stress protection exerted by Gln is thought to be due to the stimulation of autophagy in intestinal cells (Nakagawa et al., 2004; Sakiyama et al., 2009). Autophagy is the lysosome-dependent degradation of cellular proteins that is induced when cells are exposed to stresses such as a nutrient limitation, heat, oxidative challenge, and an accumulation of damaged

or excess organelles and abnormal cellular components (Yorimitsu and Klionsky, 2005). The elimination of potentially toxic components coupled with the recycling of nutrients aids in cell survival (Levine and Klionsky, 2004). Two mechanisms have been proposed for the stimulatory effect of Gln on autophagy in various cell types. One involves inhibition of the autophagy-suppressing kinase, mammalian target of rapamycin (**mTOR**; Nakajo et al., 2005; Sakiyama et al., 2009), whereas the other involves the mTOR-independent stimulation of autophagy by NH_3 liberated during glutaminolysis (Cheong et al., 2011). Intravenous infusion of Gln in calves increased intestinal glutaminolysis and NH_3 release into the portal vein feeding the liver (Nappert et al., 1999). Whether Gln or NH_3 stimulates autophagy in the liver remains unknown. The purpose of our study was to evaluate the effect of Gln on the autophagy of liver in weaned calves.

Animal care and use were approved and conducted under established standards of the College of Animal Science and Technology, China Agricultural University,

Table 1. Ingredient and nutrient composition of the experimental starter

Item	Content
Ingredient, % of DM	
Corn grain	47
Wheat bran	13
Soybean meal	20
Extruded soybeans	8.4
Corn gluten meal	7
Salt	0.55
CaCO ₃	2.1
Dicalcium phosphate	1.15
Vitamin and trace mineral premix ¹	0.8
Nutrient, % of DM	
DM, %	90.1
CP	21.25
Crude fat	4.29
NDF	16.75
ADF	6.82
Ca	1.07
P	0.56
ME, ² Mcal/kg	2.81

¹Premix contained (mg/kg): vitamin A, 4,035; vitamin D, 1,740; vitamin E, 39; Fe, 18; Zn, 37; Cu, 10.6; Mn, 15.3; Co, 0.12; I, 0.47; and Se, 0.35.

²Calculated following NRC (2001) recommendations.

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Table 2. Effects of Gln infusion on the number of autophagosomes in liver cells¹

Item	Gln, dose g/d				Effect		
	0	8	16	32	RMSE ²	Linear	Quadratic
Autophagosome, no./cell	0.5	1.5	2.1	2.5	0.25	<0.01	<0.01

¹Treatments were as follows: (1) intravenous infusion of 2 L of 0.85% NaCl (control group), (2) intravenous infusion of 8 g/d of L-Gln mixed with 2 L of 0.85% NaCl solution (L group), (3) intravenous infusion of 16 g/d of L-Gln mixed with 2 L of 0.85% NaCl solution (M group), (4) intravenous infusion of 32 g/d of L-Gln mixed with 2 L of 0.85% NaCl (H group).

²RMSE = root mean square error.

Beijing, China. Calves were randomly assigned to treatments by date of birth and were housed individually in 1.5 × 3.4 m pens inside a naturally ventilated barn. All calves received 4 L of colostrum initially, and then were fed 4 L of whole milk once daily until weaning. Fresh

calf starter (Table 1) was offered ad libitum beginning at 3 d of age. Water was offered free-choice daily. Calves were weaned at 5 wk.

Between 35 and 42 d of age, 24 calves were infused daily from 0800 to 1000 h with 2 L of 0.85% NaCl con-

Figure 1. Effects of Gln infusion on the autophagy of liver cells. Treatments were as follows: (1) intravenous infusion of 2 L of 0.85% NaCl [control (C) group]; (2) intravenous infusion of 8 g/d of L-Gln mixed with 2 L of 0.85% NaCl solution (L group); (3) intravenous infusion of 16 g/d of L-Gln mixed with 2 L of 0.85% NaCl solution (M group); (4) intravenous infusion of 32 g/d of L-Gln mixed with 2 L of 0.85% NaCl (H group). Arrows indicate autophagosomes.

taining 0 (control group), 8, 16, and 32 g/d of L-Gln. L-Glutamine was dissolved in 2 L of isotonic (0.85%) NaCl in double-distilled and pyrogen-free water, filter-sterilized through a 0.22- μ m membrane, and then stored in sterile containers. Solutions were prepared once every 3 d and kept refrigerated before use. Calves were equipped with indwelling jugular venous catheters on the first day of infusion. When not in use, the catheters were filled with 0.85% sterile saline containing 5,000 U/mL of heparin (Sigma-Aldrich Ltd., Shanghai, China) to prevent clot formation.

All calves were killed at 42 d of age. After opening the body cavity, a sample of liver was fixed with 2.5% glutaraldehyde in 100 mM PBS buffer for 12 h. After washing, the sample was postfixed with 1% OsO₄ at room temperature, dehydrated in a graded series of alcohol, and embedded in Epon 812 resin (Fluka, Buchs, Switzerland). Seventy-nanometer sections were prepared on an ultramicrotome (EM UC6, Leica, Solms, Germany) with a diamond knife, picked up on Formvar-coated grids, stained with uranyl acetate and lead citrate, and viewed under an electron microscope (H-7650, Hitachi, Tokyo, Japan). Autophagosomes were counted in 50 cells per section.

Another sample of liver was stored at -80°C until analyzed for microtubule-associated protein 1 light chain 3 (LC3) as a marker of autophagy. Frozen samples were homogenized, and proteins were separated by 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane with the Trans-Blot SD System (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with BSA and incubated overnight at 4°C with anti-LC3 (Zhongyuan Ltd., Beijing, China) and anti-actin antibodies (Sigma-Aldrich Ltd.). Membranes were then probed with secondary antibody and detected using SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific Ltd., Shanghai, China). The band density was quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

All data were analyzed as a completely randomized design using PROC GLM of SAS 9.0 (SAS Institute Inc., Cary, NC). Orthogonal polynomial contrasts were computed for linear and quadratic effects of dose. Significance was declared at $P < 0.05$.

To investigate whether infusion Gln would have effects on autophagy, the number of autophagosomes in the liver was examined by electronic microscopy. We found that infusion of Gln increased the level of autophagy in liver cells. The number of autophagosomes in the liver cells increased linearly ($P < 0.01$) with an increase in Gln infusion (Figure 1 and Table 2). From the Western blot result, we found that the abundance of LC3 increased linearly ($P < 0.05$) as the Gln infusion dose increased (Figure 2)

Autophagy was originally described as a cellular response to starvation (Nakatogawa et al., 2009). Later, autophagic induction was found to play critical roles in various cellular responses, such as prevention of infection by bacteria and viruses (Nakagawa et al., 2004; Orvedahl and Levine, 2009), early embryogenesis (Tsukamoto et al., 2008), antigen presentation (Paludan et al., 2005; English et al., 2009), neural development (Fimia et al., 2007), survival of newborns (Kuma et al., 2004), and lipid metabolism (Singh et al., 2009). Sakiyama et al. (2009) reported that Gln can induce autophagy under basal and stressed conditions. Deldicque et al. (2008) also reported that Gln has antagonistic effects on the mTOR pathway in myogenic C2C12 cells. Mammalian target of rapamycin is involved in the negative control of autophagy and has been proposed as a nutrient sensor (Kadowaki et al., 2006). Likewise, Häussinger et al. (1999) found that p38 mitogen-activated protein (MAP) kinase had a negative effect on the regulation of autophagy in hepatocytes. Comes et al. (2007) reported that Gln can induce autophagy by inhibiting the p38 MAP kinase pathway in colorectal cancer cells.

Figure 2. Effects of Gln infusion on the autophagy of liver cells. Levels of microtubule-associated protein 1 light chain 3-II (LC3-II) protein as assessed by Western blot analysis. Treatments were as follows: (1) intravenous infusion of 2 L of 0.85% NaCl [control group (C)]; (2) intravenous infusion of 8 g/d of L-Gln mixed with 2 L of 0.85% NaCl solution (L group); (3) intravenous infusion of 16 g/d of L-Gln mixed with 2 L of 0.85% NaCl solution (M group); (4) intravenous infusion of 32 g/d of L-Gln mixed with 2 L of 0.85% NaCl (H group). Hepatic LC3-II protein expression in response to Gln infusion in calves. Insets depict representative blots. Values represent means $\pm$ SE. Response from linear statistical result, $P < 0.05$. β -Actin was used to normalize the expression of target proteins.

In this experiment, we found that Gln induced autophagy in liver cells, and that the autophagy levels increased linearly as the Gln infusion dose increased. The reason may be that Gln reverses the activity of p70S6 kinase, a downstream target of mTOR, and inhibits p38 MAP kinase activity or mTOR-independent stimulation of autophagy by NH_3 liberated during glutaminolysis.

In conclusion, an exogenous supply of Gln increased the level of autophagy in the liver cells of weaned calves. To the authors' knowledge, this is the first study to report the effects of Gln on the autophagy of liver cells in the calf.

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