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Effects of L-theanine on glutamine metabolism in enterotoxigenic *Escherichia coli* (E44813)-stressed and non-stressed rats

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ABSTRACT

L-theanine, a non-protein-derived amino acid, is widely used as a functional ingredient in foods. Here, we evaluated the *in vivo* effects of L-theanine on glutamine metabolism in healthy rats and in a stressed rat model, which was established by infecting rats with enterotoxigenic *Escherichia coli* (E44813). The results showed that L-theanine significantly enhanced the synthesis of glutamine and stabilized the intestinal tract in healthy rats. In E44813-stressed rats, L-theanine could replace L-glutamine, thereby alleviating intestinal stress by reducing the expression of inflammatory factors, enhancing the activities of antioxidant enzymes, and regulating glutamine metabolism by modulating its synthesis. Treatment with a combination of L-theanine and L-glutamine was superior to treatment with L-glutamine alone in enhancing the activity of antioxidant enzymes, reducing the expression of inflammatory factors, and increasing the intestinal villi height and crypt depth.

1. Introduction

Glutamine is the most abundant free amino acid in the body, accounting for more than 60% of the total free amino acids in blood (Zhang, Pavlova, & Thompson, 2017). It is considered a "conditionally essential" amino acid and is synthesized from glutamate and ammonia through catalysis by glutamine synthetase in the skeletal muscle (Obrador et al., 2001). In a healthy individual, glutamine is the main amino acid absorbed by the small intestine and the "fuel" for intestinal epithelial cells; it is used to produce ATP that is utilized for maintaining the growth and proliferation of these cells (Souba, Herskowitz, Austgen, Chen, & Salloum, 1990). However, the small intestine must receive glutamine from endogenous or external sources because it can neither synthesize nor store glutamine. *In vivo*, glutamine is mainly derived from muscles, which are rich in glutamine synthetase and release a large amount of glutamine into the blood to provide endogenous glutamine for intestinal epithelial cells, including intestinal mucosal cells and lymphocytes (Jiang & Yu, 2000). Under stress and trauma, the demand for glutamine in the cells increases, exceeding the glutamine produced by the muscle (Petry et al., 2019), and the concentration of circulating glutamine in the body decreases by up to 30% of the concentration under normal conditions. A lack of glutamine can lead to atrophy of the intestinal mucosa, thinning and shortening of the intestinal villi, and decreased function of the intestine as a mucosal barrier in the body (Beaufrère et al., 2014; Bischoff et al., 2014; Stieler, Shannon, & Liara, 2017). Therefore, under such conditions, glutamine should be replenished from external sources. Glutamine supplementation can promote the proliferation of intestinal epithelial cells by enhancing the synthesis of proteins and reducing their degradation in these cells (Claudia, Silvia, & Guidotti, 2010; Coëffier, Claeyssens, Hecketsweiler, Lavoinne, & Ducrottã©, P., & Dã©Chelotte, P. , 2003; He et al., 2012; Wu, 2009); it also increases the villus height in the duodenum and decreases the villus height/crypt depth ratio (Dai et al., 2011; Wu, Meier, & Knabe, 1996), thereby regulating the restoration of the intestinal mucosal barrier. Glutamine can also repair this barrier under stress by increasing the expression of tight junction proteins in the intestinal mucosa (Li, Lewis, Samuelson, Liboni, & Neu, 2004; Wang et al., 2016).

Theanine is an amino acid that is mainly derived from tea. Its structure is similar to that of glutamine, and it occurs in its L-form in

Abbreviations: ETEC, enterotoxigenic; GS, glutamine synthetase; GLS, glutaminase; SOD, superoxide dismutase; CAT, catalase; GSH-PX, glutathione peroxidase; MDA, malondialdehyde; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNFα, tumor necrosis factor alpha; HE, hematoxylin and eosin

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nature. The structure of theanine, also known as γ-glutamylethylamide, is similar to that of the γ-carboxylamide group of glutamine, and theanine can be obtained by N-ethylation of glutamine. With regard to its physiological activity, L-theanine can promote the proliferation of neural stem cells and their differentiation into neurons (Takarada et al., 2016). It also influences the levels of neurotransmitters, namely acetylcholine, dopamine, and serotonin, in the brain to varying degrees (Di, Yan, & Zhao, 2012; Yao, Shen, Shen, & Wu, 2012) and inhibits cadmium-induced apoptosis in brain cells (Shen, Shen, Wang, & Wu, 2011). In the brain, L-glutamine can maintain the normal metabolism of the neurotransmitter glutamate in the central nervous system by clearing glutamate in the synaptic cleft (Hebron, Javidnia, & Moussa, 2018). Moreover, both L-theanine and L-glutamine can improve the antioxidant capacity of the body under pathological conditions and relieve inflammatory responses by downregulating the mRNA expression of inflammatory factors in the NF-κB pathway (Jing & Sun, 2011; Li et al., 2016; Li et al., 2018; Rodrigo, Jordan, Gaewyn, & Heloisa, 2019; Takagi et al., 2010; Wang et al., 2018; Wang, Wang, Li, Lu, & Liu, 2019). In addition, L-glutamine supplementation is needed to maintain plasma glutamine concentrations before long-term endurance training (Carvalhopeixoto, Alves, & Cameron, 2007; Coqueiro et al., 2018), and L-theanine can significantly improve fatigue caused by exercise (Li & Chen, 2013; Li, Shen, & Yao, 2005). Because L-theanine is similar in structure to L-glutamine and has similar physiological functions, it is of immense scientific and practical significance to study its effect on glutamine metabolism in a healthy body and to determine whether it can replace or function synergistically with L-glutamine to alleviate intestinal stress. In a healthy body, glutamine is synthesized in the skeletal muscle, released into the blood, and mainly catabolized in the small intestine.

In the present study, we studied the effects of low, medium, and high concentrations of L-theanine on glutamine synthetase activity and protein content in the skeletal muscle, glutaminase activity and protein content in the small intestine, glutamine levels in the jejunum and blood, and glutamine synthesis and metabolism in a rat model. We used enterotoxigenic (ETEC) *Escherichia coli* (E44813) to establish a rat model of intestinal stress. Based on a 7-day pre-experiment, the optimal dose of E44813 was determined to be 1.5×10^9 cfu/mL, and the intestinal stress state was maintained by feeding the rats additional bacteria every 7 days. We administered different doses of L-theanine and L-glutamine, either alone or in various combinations, to explore the metabolism of glutamine and the activities of antioxidant enzymes as well as inflammation. The results thus obtained were analyzed to determine whether L-theanine can replace or synergize with L-glutamine to exert a favorable effect on intestinal stress in rats.

2. Materials and methods

2.1. Chemicals and reagents

L-theanine (purity ≥ 98%) was purchased from Sanfu Biotechnology Co. Ltd. (Changsha, China). L-glutamine was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). ETEC strain 44,813 was provided by the China National Institutes for Food and Drug Control. The BCA protein assay kit was purchased from Solarbio Science & Technology Co. Ltd. (Beijing, China). The glutamine synthetase assay kit, glutaminase test kit, superoxide dismutase (SOD) assay kit (WST-1 method), catalase (CAT) assay kit (visible light method), glutathione peroxidase (GSH-PX) assay kit (colorimetric method), and malondialdehyde (MDA) assay kit (TBA method) were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for rat interleukin-1 beta (IL-1β), rat interleukin-6 (IL-6), and rat tumor necrosis factor alpha (TNFα) were purchased from Huamei Biotechnology Co. Ltd. (Wuhan, China). The ELISA kits for glutamine synthetase, glutaminase, and glutamine were purchased from ZCI Biotechnology Co. Ltd. (Shanghai, China).

2.2. Preparation of E44813 suspension

The E44813 suspension previously preserved in liquid nitrogen was inoculated into liquid lysogeny broth medium at a volume ratio of 1:100 and incubated in a shaking incubator at 180 rpm and 37 °C for 12 h. The cell density of the ETEC suspension was measured using an ND-1000 ultraviolet–visible (UV–Vis) spectrophotometer (NanoDrop Ltd., TX, USA) until it reached 1.5×10^9 cfu/mL. The cells were then preserved separately at 4 °C.

2.3. Animals and experimental design

Eight-week-old SD male rats (Slac Jingda Laboratory Animal Co. Ltd., Hunan, China) were used for this study. They were housed at a temperature of 25 ± 1 °C, under 12-h light conditions, and had free access to food and water. The rats were randomly divided according to weight into the following groups (10 rats in each group): Healthy group (Group O), L-theanine 300 mg/kg group (Group A), L-theanine 600 mg/kg group (Group B), L-theanine 900 mg/kg group (Group C), E44813 + L-theanine 300 mg/kg group (Group D), E44813 + L-theanine 600 mg/kg group (Group F), E44813 + L-glutamine 300 mg/kg group (Group G), E44813 + L-glutamine 600 mg/kg group (Group H), E44813 + L-glutamine 900 mg/kg group (Group I), E44813 + L-theanine 300 mg/kg + L-glutamine 600 mg/kg group (Group J), E44813 + L-theanine 450 mg/kg + L-glutamine 450 mg/kg group (Group K), and E44813 + L-theanine 600 mg/kg + L-glutamine 300 mg/kg group (Group L), E44813 group (Group M).

The rats in Groups A, B, and C were fed L-theanine at a concentration of 30, 60, and 90 mg/mL, respectively, by administering a dose of 0.1 mL/10 g body weight, and those in Group O were administered a dose of 0.1 mL/10 g body weight. The rats in the E44813-stressed group were fed a dose of E44813 at 0.1 mL/20 g body weight on the first day of every 7-day interval. At other times, rats in Group M were given normal saline at 0.1 mL/10 g body weight, and those in Groups D, E, and F were given L-theanine at concentrations of 30, 60, and 90 mg/mL, respectively, at a dose of 0.1 mL/10 g body weight. The rats in Groups G, H, and I were fed 30, 60, and 90 mg/mL L-glutamine, respectively, at a dose of 0.1 mL/10 g of body weight, whereas those in Groups J, K, and L were fed L-theanine + L glutamine at concentrations of 30 mg/mL + 60 mg/mL, 45 mg/mL + 45 mg/mL, and 60 mg/mL + 30 mg/mL at a dose of 0.1 mL/10 g body weight. After three weeks of feeding, skeletal muscles of the hind legs and jejunal segments of the small intestine were retrieved immediately after slaughter, frozen in liquid nitrogen, and then stored at −80 °C. Blood was collected and centrifuged immediately at 6000 rpm, and the supernatant was collected and stored at −80 °C. One-centimeter segments of the jejunum were fixed in paraformaldehyde for hematoxylin and eosin (HE) staining.

2.4. Histopathological examination

Histopathological examination was performed on the ileum tissue. The ileum tissues were dissected and preserved in 10% paraformaldehyde for 24 h. Thereafter, the samples were embedded in paraffin and stained with HE. All the sections were observed under a Leica light microscope.

2.5. Biochemical examination

2.5.1. Tissue pretreatment

The skeletal muscle and jejunum tissues were homogenized in normal saline [added at a ratio of 1:10 (w/v)] in an ice bath with a glass homogenizer. The homogenate was centrifuged for 10 min at 3000 rpm, and the supernatant was used for the experiments.

2.5.2. Determination of glutamine synthetase activity

Glutamine synthetase activity was determined in the skeletal muscle

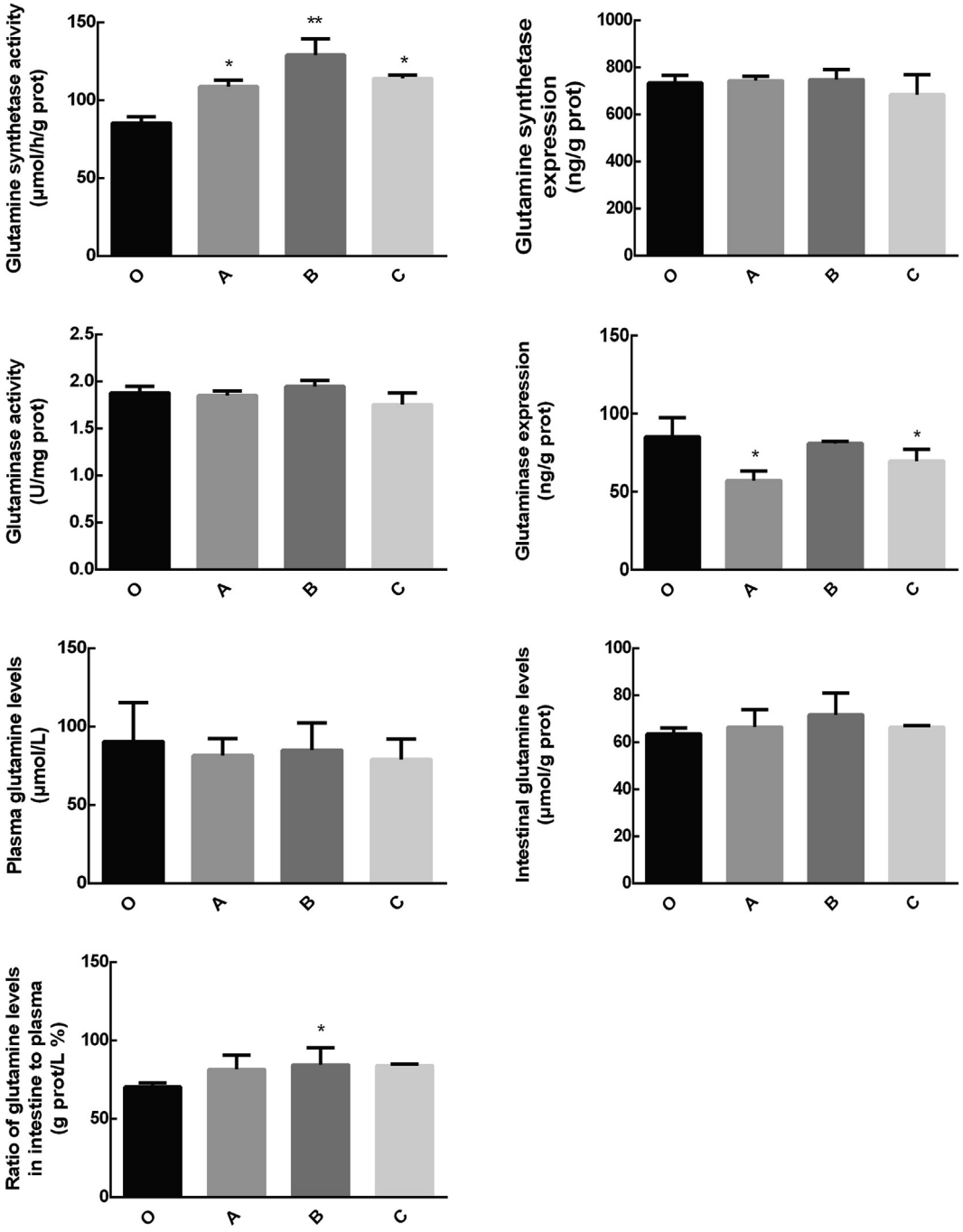

Fig. 1. Effect of L-theanine on the activity and expression of key glutamine metabolism enzymes in the skeletal muscle and intestine and on the glutamine levels in the plasma and intestine of healthy rats. Note: * A significant difference was found between the experimental and healthy groups at $P < 0.05$. ** The difference between the experimental and healthy groups was significant at $P < 0.01$. A single capital letter, such as D, indicates a significant difference between this group and Group D at $P < 0.05$. Two capital letters, such as DD, indicate a significant difference between this group and Group D at $P < 0.01$.

extract using the glutamine synthetase activity assay kit, according to the manufacturer's instructions, and was expressed as μmol/h/g protein. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of γ-glutamyl hydroxamic acid per milliliter of skeletal muscle tissue protein per milliliter of the reaction system.

2.5.3. Determination of glutamine synthetase expression

Glutamine synthetase expression was assessed in the supernatant of the skeletal muscle homogenate using an ELISA kit, according to the manufacturer's instructions, and was calculated as nanograms of glutamine synthetase protein per gram of skeletal muscle tissue protein.

2.5.4. Determination of glutaminase activity

Glutaminase activity was determined in the supernatant of the jejunum extract using a kit, according to the manufacturer's instructions, and was expressed as U/mg protein. One unit was defined as one milligram of ammonia used per milligram of intestinal tissue protein per minute to catalyze the production of glutamine.

2.5.5. Determination of glutaminase expression

Glutaminase expression was assessed in the supernatant of the jejunal extract using an ELISA kit, according to the manufacturer's instructions, and was calculated as nanograms per gram of intestinal

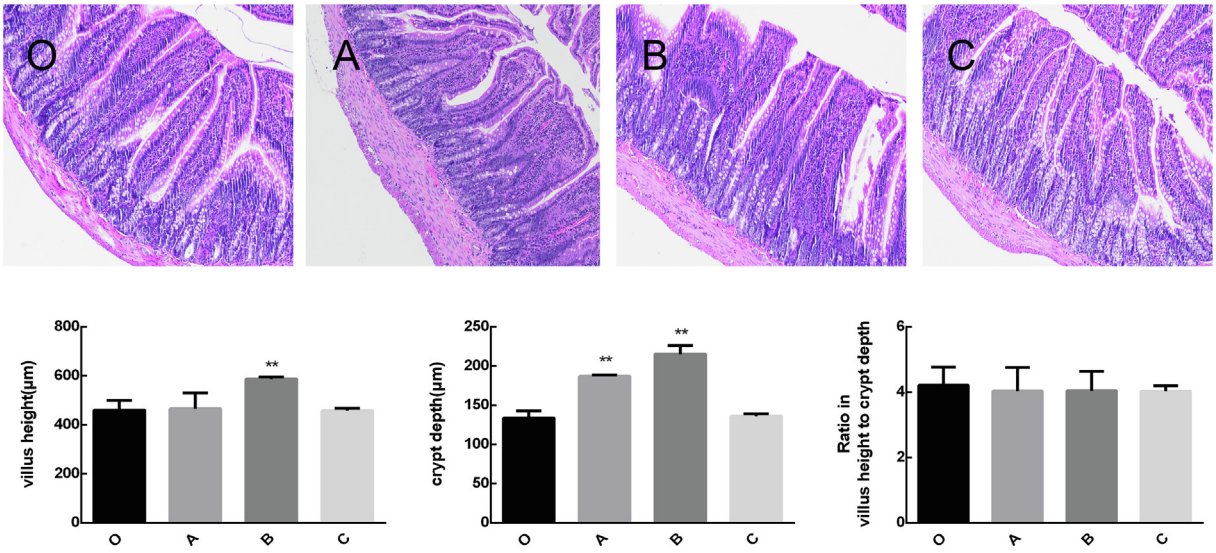

Fig. 2. Effect of L-theanine on the intestinal morphology in healthy rats. Note: * A significant difference was found between the experimental and healthy groups at $P < 0.05$. ** The difference between the experimental and healthy groups was significant at $P < 0.01$. A single capital letter, such as D, indicates a significant difference between this group and Group D at $P < 0.05$. Two capital letters, such as DD, indicate a significant difference between this group and Group D at $P < 0.01$.

tissue protein.

2.5.6. Determination of glutamine levels

Glutamine levels were determined in plasma and in the supernatant of the jejunal extract using an ELISA kit, according to the manufacturer's instructions, and the levels were expressed as μmol/mL of plasma or μmol/g of intestinal tissue protein.

2.5.7. Determination of CAT activity

CAT activity was determined in plasma using the CAT assay kit, according to the manufacturer's instructions, and was expressed as U/mL. One unit was defined as the amount of enzyme that decomposed 1 μmol of H_2O_2 per milliliter of serum per second.

2.5.8. Determination of SOD activity

SOD activity in plasma was determined using the SOD assay kit, according to the manufacturer's instructions, and was expressed as U/mL. One unit of activity was defined as the amount of SOD at which 50% of an oxidant was inhibited in the reaction system.

2.5.9. Determination of GSH-PX activity

GSH-PX activity in plasma was measured using the GSH-PX activity kit, according to the manufacturer's instructions. One unit of GSH-PX activity was defined as the amount of enzyme required to reduce the GSH concentration (assuming no enzymatic action) by 1 μmol/L in 0.1 mL of serum in the reaction solution in 5 min.

2.5.10. Determination of MDA content

MDA content in plasma was measured using the MDA content kit, as described by the manufacturer, and was expressed as nanomoles of MDA per milliliter of plasma.

2.5.11. Determination of the expression levels of IL-1β, IL-6, and TNF-α

The expression levels of IL-1β, IL-6, and TNF-α were determined using the respective ELISA kits, according to the manufacturer's instructions, and were calculated as picograms per milligram of intestinal tissue protein.

2.6. Statistical analysis

Image-Pro Plus 6.0 was used to measure the villus length and crypt depth of the jejunum in HE-stained sections, and GraphPad Prism 6 software was used for graphic analysis. SPSS 16.0 statistical software was used for one-way ANOVA, and the Student–Newman–Keuls post hoc test was used for inter-group comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Regulation of glutamine metabolism in healthy rats by L-theanine

The activity of glutamine synthetase in the skeletal muscle was increased significantly after administration of L-theanine (Fig. 1). The effect on glutamine synthetase activity in the skeletal muscle was most prominent in Group B, compared to the effects in Groups A and C. This is because the amount of glutamate produced by L-theanine decomposition at a low concentration is lower than that produced at a medium concentration. Thus, the amount of substrate for glutamate synthesis in the skeletal muscle was lower than that in Group B, and the glutamate synthetase activity in Group A was lower than that in Group B. In addition, the affinity of L-theanine for the glutamate transporter is lower than that of glutamate (Unno, Suzuki, Kakuda, Hayakawa, & Tsuge, 1999); at medium and low concentrations, L-theanine does not have any obvious competitive effect on the glutamate transporter. At a higher L-theanine concentration, although more glutamate is produced, the glutamate transporter is competitively inhibited, reducing the glutamate transported to the skeletal muscle. Therefore, the glutamine synthetase activity in the skeletal muscle of rats in Group C was lower than that in Group B. The results indicated that 600 mg/kg L-theanine had the best effect in promoting glutamine anabolism. The change in the expression of glutaminase protein in the intestinal tract was due to the breakdown of glutamine by glutaminase in the intestinal tract and the release of ATP, which provides energy for physiological activities in the intestine (Darmaun, 1993), keeping the ATP levels stable. The total glutaminase activity remained unchanged, and the total amount of glutaminase protein was decreased, indicating that L-theanine could promote the translation and modification of glutaminase protein and improved the ability of the intestinal tract to withstand the trauma caused by stress.

After the administration of L-theanine, the glutamine content in plasma tended to decrease, the intestinal glutamine content increased, and the ratio of intestinal to plasma glutamine was significantly higher

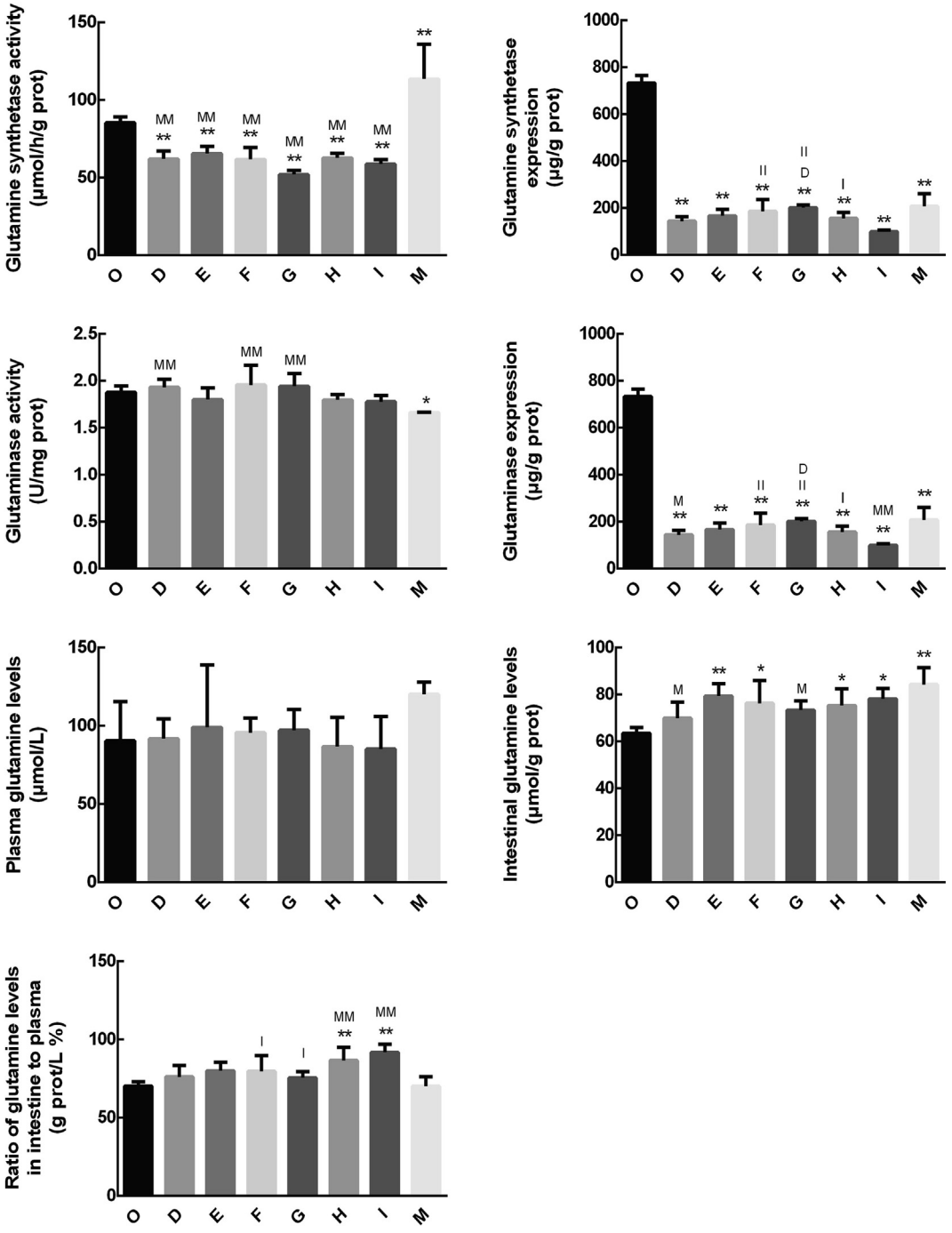

Fig. 3. Effect of L-theanine on the activity and expression of key glutamine metabolism enzymes in the skeletal muscle and intestine and on the glutamine levels in the plasma and intestine of E44813-stressed rats. Note: * A significant difference was found between the experimental and healthy groups at $P < 0.05$. ** The difference between the experimental and healthy groups was significant at $P < 0.01$. A single capital letter, such as D, indicates a significant difference between this group and Group D at $P < 0.05$. Two capital letters, such as DD, indicate a significant difference between this group and Group D at $P < 0.01$.

than in the healthy group (Fig. 2). L-theanine is suggested to improve the ability of intestinal epithelial cells to take up glutamine from the blood; this effect might be related to the glutamine transporter in the intestinal epithelial cell membrane (Wagenmakers, 1992). Based on the current understanding, glutamine has an important effect on the intestinal structure. In this study, we found that L-theanine is beneficial for intestinal absorption of glutamine. Therefore, we further studied whether L-theanine could affect the intestinal structure by regulating glutamine metabolism.

As observed in the stained intestinal sections, the intestinal mucosal layer, mucosal muscle layer, submucosal layer, and muscle layer in the intestinal tissues in Groups O, A, B, and C had a distinct structure, with intact villous epithelium and a fine structure of goblet cells, and the tissue morphology was normal (Fig. 3). Comparative analyses of Groups O, A, B, and C revealed that the intestinal villus length and crypt depth were increased after feeding L-theanine, and this trend was consistent with the change in intestinal glutamine concentration after the feeding of L-theanine. Crypt cells, which are the proliferating cells in the intestine, can differentiate into villus cells in the small intestine (Darmaun, 1993). Studies have shown that glutamine can promote the proliferation of crypt cells (Wu, 2009); therefore, we inferred that they play an important role in absorption by the small intestine and in

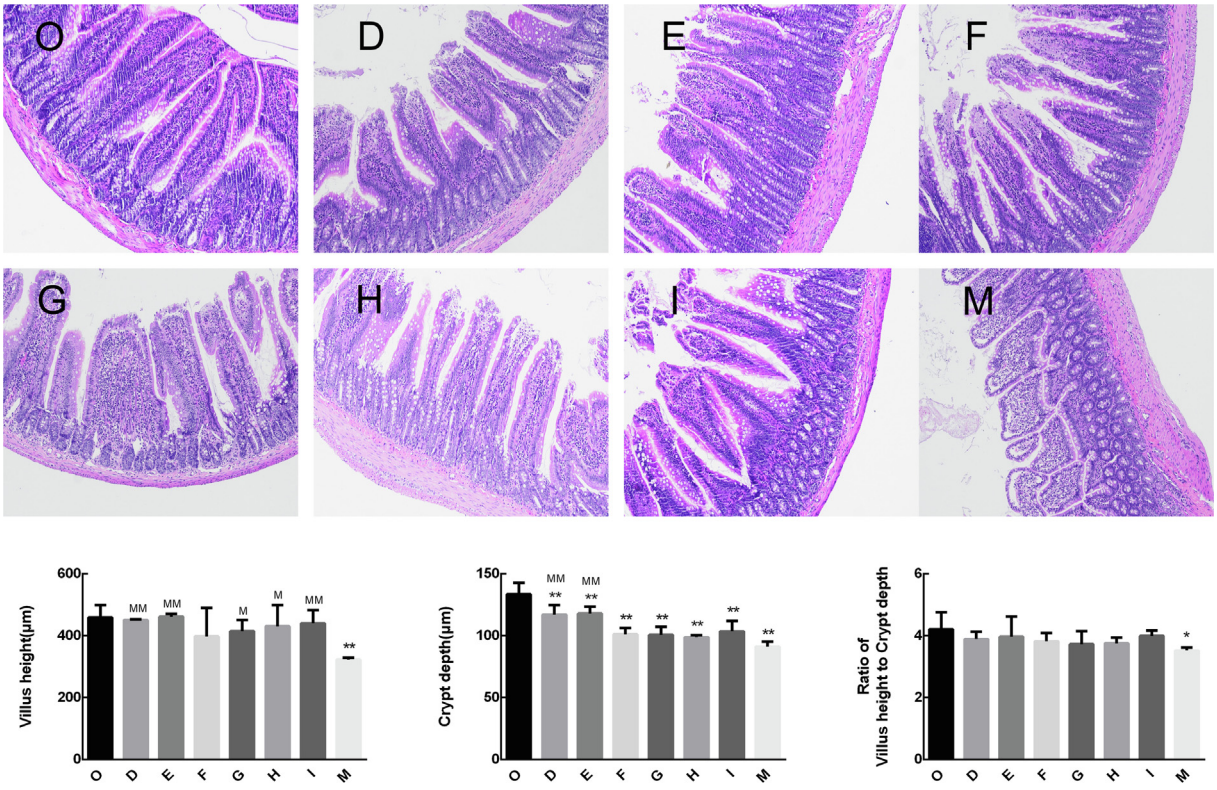

Fig. 4. Effect of L-theanine on the intestinal morphology in E44813-stressed rats. Note: * A significant difference was found between the experimental and healthy groups at $P < 0.05$. ** The difference between the experimental and healthy groups was significant at $P < 0.01$. A single capital letter, such as D, indicates a significant difference between this group and Group D at $P < 0.05$. Two capital letters, such as DD, indicate a significant difference between this group and Group D at $P < 0.01$.

maintaining intestinal stability. It is suggested that L-theanine regulates the structure and absorption capacity of the intestinal tract by increasing the concentration of glutamine absorbed from the blood to the intestinal tract in a healthy body.

3.2. Regulation of glutamine metabolism by L-theanine in E44813-stressed rats

As shown in Figs. 4 and 5, the intestines of Group M rats were damaged after feeding E44813, and the bodily need for glutamine was increased sharply (Kim, Li, Jang, & Arany, 2017); thus, as a compensatory effect, the activity of glutamine synthetase in the skeletal muscle was increased, and the amount of glutamine released into the blood was increased. However, the increase in TNF-α levels inhibited protein synthesis in the skeletal muscle, which reduced the amount of glutamine synthetase in the skeletal muscle; these results are consistent with those reported previously (Zhou, Li, Liu, Cao, & Li, 2007). The ratio of glutamine in the intestine to that in plasma did not change compared to that in Group O, indicating that the intestinal absorption of glutamine from the blood was not increased. E44813 causes an obvious inflammatory response as well as intestinal edema and cell degeneration, interfering with absorption and secretion by intestinal epithelial cells (Luise et al., 2019). In the case of intestinal injury, intake of L-theanine and L-glutamine can significantly relieve inflammation, improve the activity of intestinal glutamine enzyme, and improve the absorption and utilization of intestinal glutamine. Therefore, both L-theanine and L-glutamine can repair intestinal injury by lowering the levels of inflammatory cytokines and restore the intestinal absorption capacity by increasing the intestinal villi length and crypt depth. Exogenously supplemented L-glutamine is directly absorbed by intestinal epithelial cells and is used for providing energy to these cells as well as for repair of the intestinal tract (Yao et al., 2012). Exogenously supplemented L-theanine is absorbed by the body and is cleaved into glutamate and ethylamine in the kidney after transport through the blood; it is then converted into glutamine by glutamine synthetase. As shown in Fig. 4, L-theanine intake reduced the activity of glutaminase in the skeletal muscle compared to that in Groups O and M; this effect was consistent with that of L-glutamine intake. After intake of L-theanine by healthy organisms, glutamine synthetase activity in the skeletal muscle is significantly increased because glutamine is the main energy source for cells of the intestinal tract and is synthesized and released by skeletal muscle. Although the liver contains both glutaminase and glutamine synthetase, it only maintains the glutamine concentration in the blood (Elia, Folmer, Schlatmann, Goren, & Austin, 1989; Wagenmakers, Coakley, & Edwards, 1990). Therefore, in a healthy body, L-theanine affects glutamine synthesis in the skeletal muscle. When the body is stressed, glutamine catabolism is enhanced, the concentration and direction of glutamine flow in the liver are changed, synthesis of urea is inhibited, and glutamine synthetase activity is enhanced, promoting the synthesis of glutamine in the liver; these changes can alleviate the degradation of skeletal muscle proteins (Elia et al., 1989; Wagenmakers et al., 1990). Therefore, when the body is stressed by E44813, glutamate produced by the decomposition of L-theanine is no longer transported to the skeletal muscle for synthesis, and the kidneys or liver can directly synthesize glutamine (Parry-Billings et al., 1992).

The intestinal villus length and crypt depth in E44813-stressed rats were significantly increased after L-theanine and L-glutamine intake ($P < 0.05$), and the ratio of villus length to crypt depth was also increased, indicating that L-theanine and L-glutamine can alleviate intestinal stress in E44813-stressed rats to a certain extent by repairing the intestinal morphology and by restoring the nutrient absorption capacity of the intestine (Hübl, Druml, Roth, & Lochs, 1994).

We observed that rats in Group M exhibited severe oxidative damage as a result of the intestinal stress induced by E44813 (Table 1).

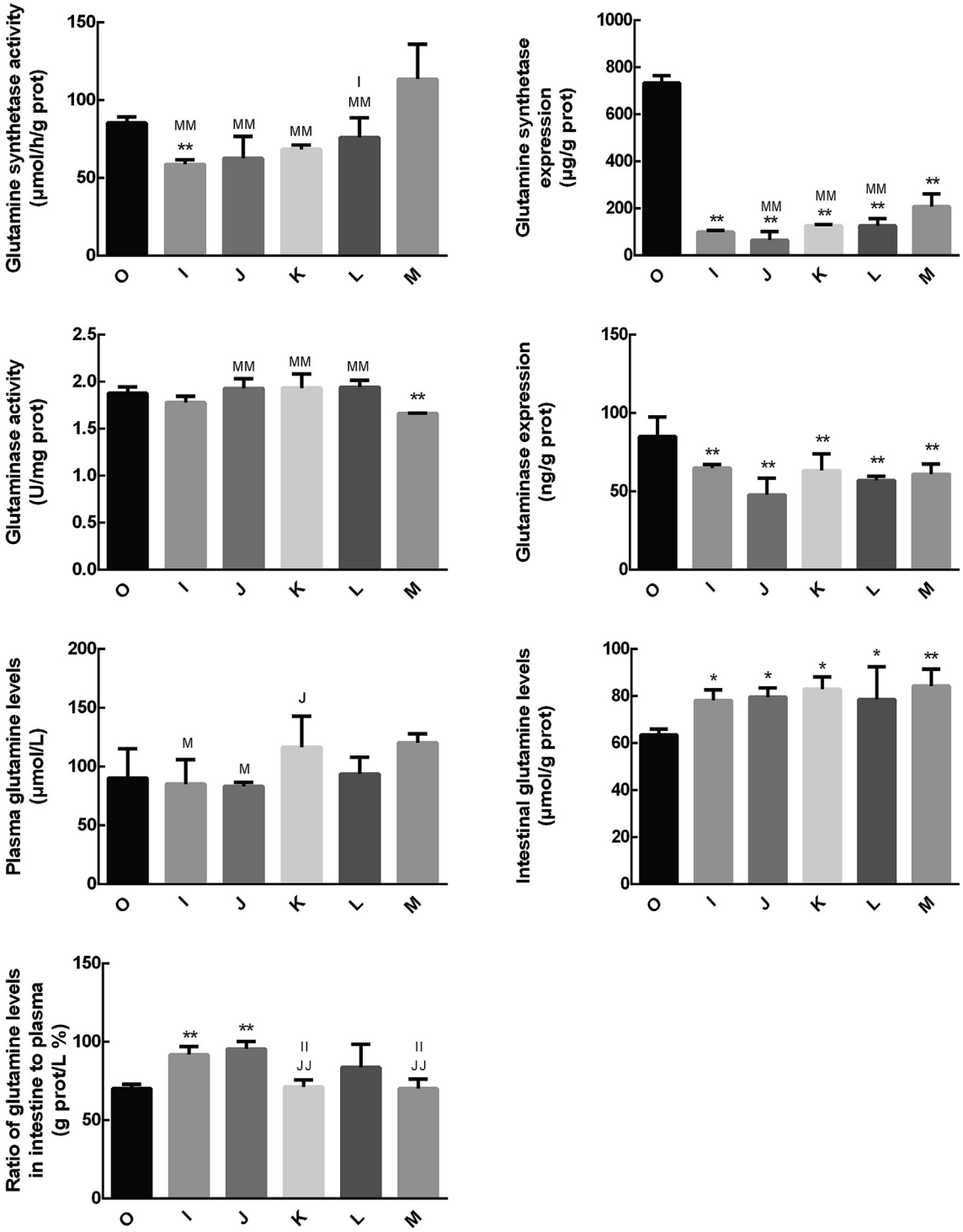

Fig. 5. Effect of administering a combination of L-theanine and L-glutamine on the activity and expression of key glutamine metabolism enzymes in the skeletal muscle and intestine and on the glutamine levels in the plasma and intestine of E44813-stressed rats. Note: * A significant difference was found between the experimental and healthy groups at $P < 0.05$. ** The difference between the experimental and healthy groups was significant at $P < 0.01$. A single capital letter, such as D, indicates a significant difference between this group and Group D at $P < 0.05$. Two capital letters, such as DD, indicate a significant difference between this group and Group D at $P < 0.01$.

The antioxidant system in the rats was destroyed, and the activities of CAT, SOD, and GSH-PX (Dong, Wang, Qin, Cao, & Chen, 2018), which can effectively eliminate harmful substances (peroxides, free radicals, etc.) from cells, were significantly decreased. Moreover, the content of one of the most important products of membrane lipid peroxidation, MDA, was increased significantly. On the contrary, the levels of IL-1β, IL-6, and TNF-α were increased significantly in Group M after administration of E44813. Excessive cytokine levels are responsible for severe immune response, resulting in toxic effects and inflammation (Demir et al., 2013). After feeding L-theanine or L-glutamine to rats subjected to intestinal stress, the activities of antioxidant enzymes and intestinal inflammatory response were changed; in particular, the levels of inflammatory factors were significantly reduced compared to those in Group M, suggesting that L-theanine and L-glutamine could

Table 1
Effects of L-theanine on intestinal oxygen factors and inflammatory factors in E44813-stressed rats.

	CAT activity (U/mL)	SOD activity (U/mL)	GSH-PX activity (U)	MDA content (nmol/mL)	Expression levels (pg/mg prot)		
					IL-1β	IL-6	TNF-α
O	43.06 ± 7.30	86.79 ± 1.76	1375.14 ± 54.27	9.59 ± 0.47	55.68 ± 25.89	0.27 ± 0.03	6.27 ± 3.03
D	29.81 ± 8.65*	91.47 ± 4.81	1321.62 ± 101.40	11.10 ± 0.24*	139.06 ± 24.34^{MM}	0.34 ± 0.08	8.55 ± 1.87
E	37.11 ± 5.98	94.23 ± 1.76*	1336.22 ± 60.24	10.82 ± 0.89	91.33 ± 32.45 ^{MM}	0.32 ± 0.03	6.93 ± 1.36 ^{MM}
F	31.24 ± 9.27*	86.71 ± 0.45	1550.27 ± 18.49**	10.82 ± 0.66	106.84 ± 14.04 ^{M}	0.43 ± 0.26	7.43 ± 1.43 ^{M}
G	32.52 ± 5.68	82.42 ± 3.77^{DD}	1459.46 ± 81.08	11.40 ± 0.69**	114.12 ± 66.78 ^{M}	0.64 ± 0.34^{**,DD}	7.89 ± 1.27 ^{M}
H	26.50 ± 6.97**	82.71 ± 1.72^{EE}	1467.57 ± 73.06^{EE}	11.51 ± 0.52**	165.98 ± 41.69*	0.37 ± 0.08	6.87 ± 1.23 ^{MM}
I	28.23 ± 1.03*	84.55 ± 5.41	1462.16 ± 70.62 ^{MM}	11.88 ± 1.48**	101.88 ± 19.65 ^{MM}	0.35 ± 0.10	6.28 ± 1.18 ^{MM}
M	24.54 ± 1.97**	80.29 ± 3.26*	1253.15 ± 74.13*	13.38 ± 0.89**	237.86 ± 123.56**	0.67 ± 0.02**	11.60 ± 4.27**

Note: * A significant difference was found between the experimental and healthy groups at $P < 0.05$. ** The difference between the experimental and healthy groups was significant at $P < 0.01$. A single capital letter, such as D, indicates a significant difference between this group and Group D at $P < 0.05$. Two capital letters, such as DD, indicate a significant difference between this group and Group D at $P < 0.01$.

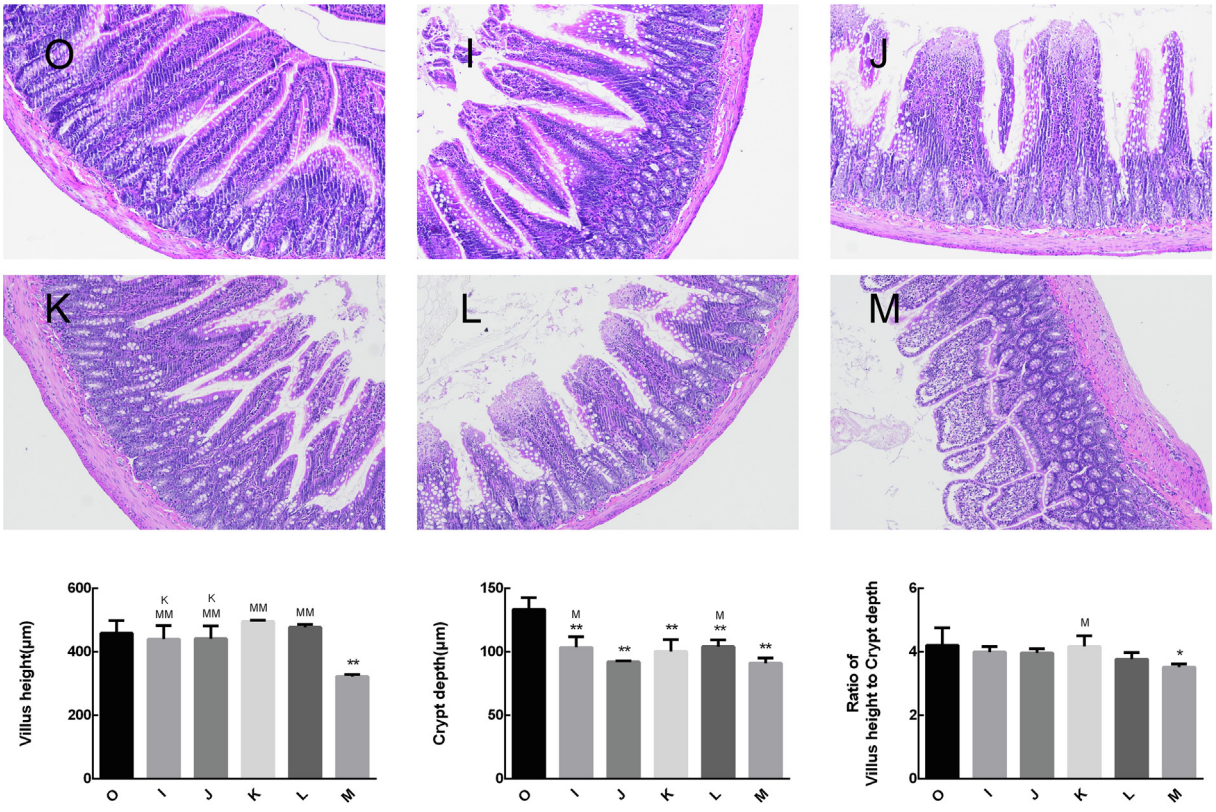

Fig. 6. Effect of administering a combination of L-theanine and L-glutamine on the intestinal morphology in E44813-stressed rats. Note: * A significant difference was found between the experimental and healthy groups at $P < 0.05$. ** The difference between the experimental and healthy groups was significant at $P < 0.01$. A single capital letter, such as D, indicates a significant difference between this group and Group D at $P < 0.05$. Two capital letters, such as DD, indicate a significant difference between this group and Group D at $P < 0.01$.

Table 2
Effects of administering a combination of L-theanine and L-glutamine on intestinal oxygen factors and inflammatory factors in E44813-stressed rats.

	CAT activity (U/mL)	SOD activity (U/mL)	GSH-PX activity (U)	MDA content (nmol/mL)	Expression levels (pg/mg prot)		
					IL-1β	IL-6	TNF-α
O	43.06 ± 7.30	86.79 ± 1.76	1375.14 ± 54.27	9.59 ± 0.47	55.68 ± 25.89	0.27 ± 0.03	6.27 ± 3.03
I	28.23 ± 1.03*	84.55 ± 5.41	1462.16 ± 70.62 ^{MM}	11.88 ± 1.48**	101.88 ± 19.65 ^{MM}	0.35 ± 0.10	6.28 ± 1.18 ^{MM}
J	25.72 ± 7.62*	90.73 ± 5.26 $^{MM, I}$	1516.22 ± 97.16 ^{MM}	11.87 ± 0.62^{**,M}	110.54 ± 16.39 ^{M}	0.55 ± 0.24**	8.67 ± 1.44
K	36.66 ± 13.33	92.97 ± 2.79 ^{MM,II}	1440.54 ± 51.71 ^{MM}	12.05 ± 0.77^{**,M}	159.88 ± 65.38	0.54 ± 0.11*	8.87 ± 1.25
L	31.39 ± 2.99	97.97 ± 4.57^{**,MM,JJ,II}	1394.59 ± 161.51*, ^{MM}	10.78 ± 0.44^{MM}, ^{K}	103.87 ± 5.02 ^{M}	0.36 ± 0.12 ^{MM}	8.94 ± 1.10
M	24.54 ± 1.97**	80.29 ± 3.26*	1253.15 ± 74.13*	13.38 ± 0.89**	237.86 ± 123.56**	0.67 ± 0.02**	11.60 ± 4.27**

Note: * A significant difference was found between the experimental and healthy groups at $P < 0.05$. ** The difference between the experimental and healthy groups was significant at $P < 0.01$. A single capital letter, such as D, indicates a significant difference between this group and Group D at $P < 0.05$. Two capital letters, such as DD, indicate a significant difference between this group and Group D at $P < 0.01$.

effectively alleviate the inflammatory response of organisms facing intestinal stress. Comparison of SOD activity and IL-6 expression in Group D and G rats showed that L-theanine had a better effect in improving antioxidant enzyme activity and in reducing inflammation than L-glutamine when used at the same concentration (300 mg/kg). This result demonstrated that similarly to L-glutamine, L-theanine alleviates the inflammatory reaction caused by oxidative stress and protects against intestinal damage in E44813-stressed rats; however, the optimal concentrations of L-theanine (600 mg/kg) and L-glutamine (900 mg/kg) are not the same, and a lower dose of L-theanine, compared to that of L-glutamine, would be required to relieve the same degree of E44813 stress.

3.3. Synergistic effect of L-theanine and L-glutamine on the regulation of glutamine metabolism in E44813-stressed rats

The combination of L-theanine and L-glutamine increased the levels of glutamine in the blood and intestinal tract of E44813-stressed rats, indicating that the combined intake of L-theanine and L-glutamine repaired the intestinal capacity of glutamine absorption from food as well as enhanced glutamine metabolism in the body (Fig. 5). The combination of L-theanine and L-glutamine had a better effect on glutamine synthesis in rats subjected to intestinal stress compared to the effect of the same dose of L-glutamine (Fig. 5). The glutamine synthetase activity in skeletal muscle increased with the increase in the ratio of L-theanine in the combined treatment. Moreover, the activity of intestinal glutaminase was increased after administering L-theanine and L-glutamine in combination, indicating that this combination can strengthen the catabolism and improve the utilization of glutamine in the intestinal tract.

The combination of L-theanine and L-glutamine had a better effect on the intestinal structure in E44813-stressed rats compared to L-glutamine treatment alone (Fig. 6). The increase in the villus length in Group K rats (450 mg/kg of L-theanine + 450 mg/kg of L-glutamine) was significantly higher than that in Group I rats (900 mg/kg of L-glutamine). This indicated that L-theanine has a synergistic effect with L-glutamine that protects the body from intestinal stress and can enhance the protective effect of L-glutamine against intestinal stress.

The combination of L-theanine and L-glutamine also significantly increased the activity of SOD and GSH-PX in E44813-stressed rats ($P < 0.05$), and reduced the MDA content ($P < 0.05$) and expression of inflammatory factors (Table 2). In terms of improving the antioxidant enzyme activity in E44813-stressed rats, the effect of the combination treatment was generally better than that of the high dose (900 mg/kg) of L-glutamine alone; in particular, the SOD activity in Groups J, K, and L was significantly higher than that in Group I ($P < 0.05$). The results indicated that L-theanine relieved the intestinal oxidative stress in E44813-stressed rats in coordination with L-glutamine and improved the ability of glutamine to relieve oxidative stress. In this study, L-theanine was found to promote glutamine metabolism by increasing the activity of glutamine synthetase in the skeletal muscle of healthy rats; cleavage of L-theanine can increase the concentration of glutamate, which is the substrate for glutamine synthesis (Zhu et al., 2019). For L-theanine, 600 mg/kg was found to be the optimal dose because at 300 mg/kg, cleavage of L-theanine produced a lower amount of glutamate, and at 900 mg/kg, L-theanine competitively inhibited the glutamate transporters and reduced the amount of glutamate transported to the skeletal muscle. According to the equivalent dose-volume parameters described in the *Experimental Methodology of Pharmacology* (Xu, 2010), the dose administered to rats should be 6.3 times that administered to humans. Therefore, for 600 mg/kg L-theanine, the equivalent optimal dose for humans would be about 95 mg/kg.

In E44813-stressed rats, the effects of the medium L-theanine dose (600 mg/kg) on the activity of antioxidant enzymes, expression levels of inflammation factors, and repair of intestinal structure were comparable to those of the high dose of L-glutamine (900 mg/kg) because the intake of L-theanine improved the glutamine synthesis ability of the body. Moreover, L-theanine itself has antioxidant and anti-inflammatory activities. Therefore, the combination of L-theanine and L-glutamine had better effects on the antioxidant enzyme activity, expression of inflammatory factors, and intestinal structure in E44813-stressed rats than L-glutamine (900 mg/kg) alone because the combination treatment supplemented L-glutamine exogenously and repaired the metabolic machinery for the synthesis of endogenous glutamine. However, the specific mechanism underlying the effect of L-theanine on glutamine metabolism in the body, such as the signaling pathway involved, has not been thoroughly investigated and warrants further study. Nonetheless, L-theanine has good antioxidant and anti-inflammatory effects, which are enhanced when used in combination with L-glutamine. The synergistic effect of L-theanine and other substances, especially catechins, caffeine, and polysaccharides present in tea, should be studied further. Evaluation of the effects of the combination treatments compared with L-theanine alone could provide a theoretical basis for the development of natural compounds as therapeutic drugs.

4. Conclusion

This study demonstrated that L-theanine can significantly improve the synthesis and utilization of glutamine *in vivo* and stability of the intestinal structure in healthy rats. In E44813-stressed rats, L-theanine could replace L-glutamine, thereby alleviating the intestinal stress to some extent, and modulate glutamine metabolism by regulating the synthesis of glutamine. However, a combination of L-theanine and L-glutamine had a synergistic effect in improving the intestinal stability and antioxidant capacity and in reducing the inflammatory response to intestinal stress caused by E44813.

5. Ethics statements

All experimental procedures and animal care were performed in strict accordance with the Animal Care and Use Guidelines and the Ethics Committee of Hunan Agriculture University (registry number:015063506).

Declaration of Competing Interest

Authors declare no conflicts of interest.

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