Animal Nutrition 7 (2021) 1031–1038

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

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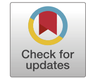
Original Research Article

The in ovo injection of methionine improves intestinal cell proliferation and differentiation in chick embryos by activating the JAK2/STAT3 signaling pathway



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A R T I C L E I N F O

Article history:
Received 18 October 2020
Received in revised form
25 January 2021
Accepted 9 March 2021
Available online 31 July 2021

Keywords:
In ovo injection
Chick
Intestine
L-methionine
JAK2/STAT3

A B S T R A C T

The intestinal health of chick embryos is vital for their life-long growth, and exogenous nutrition intervention may provide sufficient nutrition for embryonic development. In the present study, we investigated the effect of in ovo injection of L-methionine (L-Met) on the intestinal structure and barrier function of chick embryos. There were 4 groups of treatments: the control (CON) group injected with phosphate-buffered saline (PBS) and the other 3 groups injected with 5, 10, and 20 mg L-Met/egg, respectively. The injection was performed on embryonic day 9 (E9), and intestinal samples were collected on the day of hatching for analysis. The results showed that, compared with the CON group, the groups administered an in ovo injection of L-Met increased relative weights of the duodenum, jejunum, and ileum ($P < 0.05$). Hematoxylin and eosin (H&E) staining showed that the groups injected with 5, 10, and 20 mg L-Met significantly increased villus height and crypt depth ($P < 0.05$). Moreover, in ovo injection of 10 mg L-Met also increased the transepithelial electrical resistance (TEER) of the jejunum ($P < 0.05$). Injection with 10 and 20 mg L-Met increased the expression of the tight junction proteins (ZO-1 and claudin-1) and the fluorescence signal intensity of Ki67 and villin proteins ($P < 0.05$). Further, the protein expression of phospho-Janus kinase 2 (p-JAK2) and phospho-signal transducer and activator of transcription 3 (p-STAT3) was significantly increased by 10 or 20 mg L-Met injection ($P < 0.05$). In conclusion, the injection of L-Met, especially at a dose of 10 mg, showed beneficial effects on the intestinal integrity of chick embryos due to the activation of the JAK2/STAT3 signaling pathway. Our results may provide new insights for regulating the intestinal development of embryonic chicks and the rapid growth of chicks after hatching.

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1. Introduction

The small intestinal epithelium is the major site of nutrient digestion and absorption, and it also provides a natural defense for chicks against pathogens (Peterson and Artis, 2014; Gilbert et al., 2007). To our knowledge, the chick intestine is visible at approximately embryonic day 5 (E5) and then develops continuously to form a typical structure with a crypt–villus axis until the end of hatching (Southwell, 2006). It is noteworthy that the gut grows rapidly in the late hatching period with the marked consumption of egg nutrients, which limits intestinal development and embryo growth (Southwell, 2006; Li et al., 2008; Moran, 2007). Our previous study confirmed that the yolk of chick embryos was completely enclosed by the yolk membrane at E9, which provided reliable time points for nutrition intervention in embryo eggs (Chen et al., 2020a). Therefore, supplementation with exogenous nutrients into eggs may be beneficial for the intestinal development of embryonic chicks and for the rapid growth of chicks after hatching (Willemsen et al., 2010; Kadam et al., 2013).

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

https://doi.org/10.1016/j.aninu.2021.03.009
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Dietary amino acids are crucial for the gut to potentiate its functions and prevent diseases (Wang et al., 2009). As the first-limiting amino acid in diets for poultry, methionine (Met) has been demonstrated to increase chick weight (Vieira et al., 2004). Moreover, Met is a key regulator of embryonic stem cell differentiation (Shiraki et al., 2014). A previous report involving essential amino acids in enteral nutrition in mice identified that Met deprivation affects the proliferation and differentiation of intestinal stem cells (ISC), which are located at the bottom of the crypts and drive intestinal epithelial renewal and regeneration (Saito et al., 2017). Consistently, supplementation with both Met and its hydroxyl analogs improves intestinal epithelial integrity by promoting the rapid growth of mouse ISC in the context of deoxynivalenol stimulation (Zhou et al., 2019). However, it remains to be further investigated whether the in ovo administration of Met contributes to the intestinal development of chick embryos under homeostasis.

The Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway play a pivotal role in the homeostasis and regeneration of intestinal epithelium (Jiang et al., 2009). Mechanistically, STAT3 is recruited to the activated cytokine receptor and then tyrosine-phosphorylated by receptor-associated JAK2 (Tang et al., 2018). Several studies have confirmed STAT3 as a transcription factor that induces the expression of specific genes, such as cyclin D1, and that is also involved in the transcription of tight junction gene, which regulates the processes of enterocyte proliferation and barrier construction respectively (Yu et al., 2009; Morikawa et al., 2011). Furthermore, STAT3 activation protects the intestinal integrity of mice by accelerating the regeneration of ISC (Hou et al., 2018). Interestingly, Obata et al. (2018) found that S-adenosylmethionine (SAM), the first metabolite of Met, induced ISC division via JAK/STAT. Nevertheless, the mechanism by which Met maintains the integrity of the chick intestinal epithelium, especially the regulation of intestinal cell activity, is still unclear.

Here, we explored the effects of in ovo L-Met injection on the intestinal development of chicks at E9. We demonstrate that L-Met promotes intestinal cell proliferation to improve intestinal epithelial integrity by activating the JAK2/STAT3 pathway. Our findings might provide a nutritional intervention for chick embryo growth involving JAK2/STAT3 signaling.

2. Materials and methods

2.1. Animal ethics

The experiments were approved by the Care and Use of Laboratory Animals Committee of South China Agricultural University (Guangzhou, Guangdong, China).

2.2. L-Met solutions and in ovo injection procedure

L-Met purchased from Sigma–Aldrich (purity ≥ 98%, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) and filtered using a 0.22-μm syringe filter.

The fertilized eggs of yellow-feathered broiler chicks, purchased from Qingyuan Chicken Company (Qingyuan, Guangdong, China), were incubated in an automatic hatcher (CFK, Keyu Incubation Equipment Company, Dezhou, Shandong). On E6 unfertilized and dead eggs were removed; then, a total of 80 eggs (45.03 ± 2.25 g) were selected and randomly divided into 4 treatments with 20 eggs per treatment. PBS (control, CON), 5, 10, or 20 mg L-Met was added to the eggs by in ovo injection at E9. The steps were as follows: firstly, a needle was used to make a small hole at 1/3 of the sharp end of the embryo egg after disinfection with a 75% alcohol-soaked cotton ball; secondly, 0.5 mL of the solution was injected into the yolk with a 1 mL disposable sterile syringe. After hatching, the chicks were sacrificed by CO_2 inhalation followed by cervical dislocation to ensure death, then the intestinal samples were collected.

2.3. Hematoxylin and eosin (H&E) staining

After fixing with 4% paraformaldehyde, the jejunum samples were made into paraffin-embedded tissues for further histological

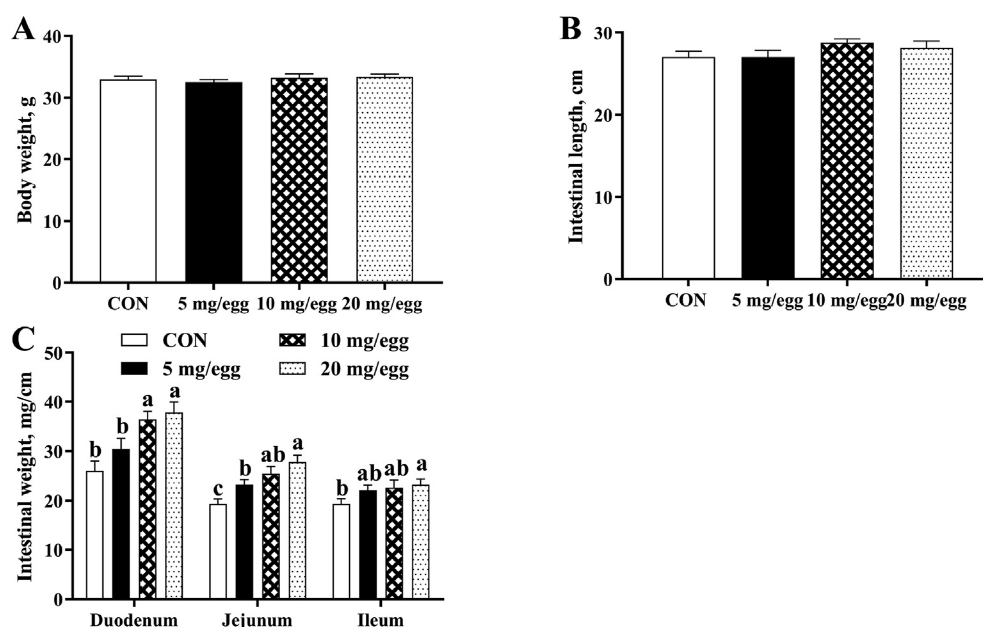

Fig. 1. The in ovo injection of methionine increases the small intestine weight of chicks. (A) Body weight. (B) Intestinal weight. (C) Intestinal length. Bars without a common letter indicate significant differences ($P < 0.05$); the values are means ± SEM. $n = 6$. CON = control.

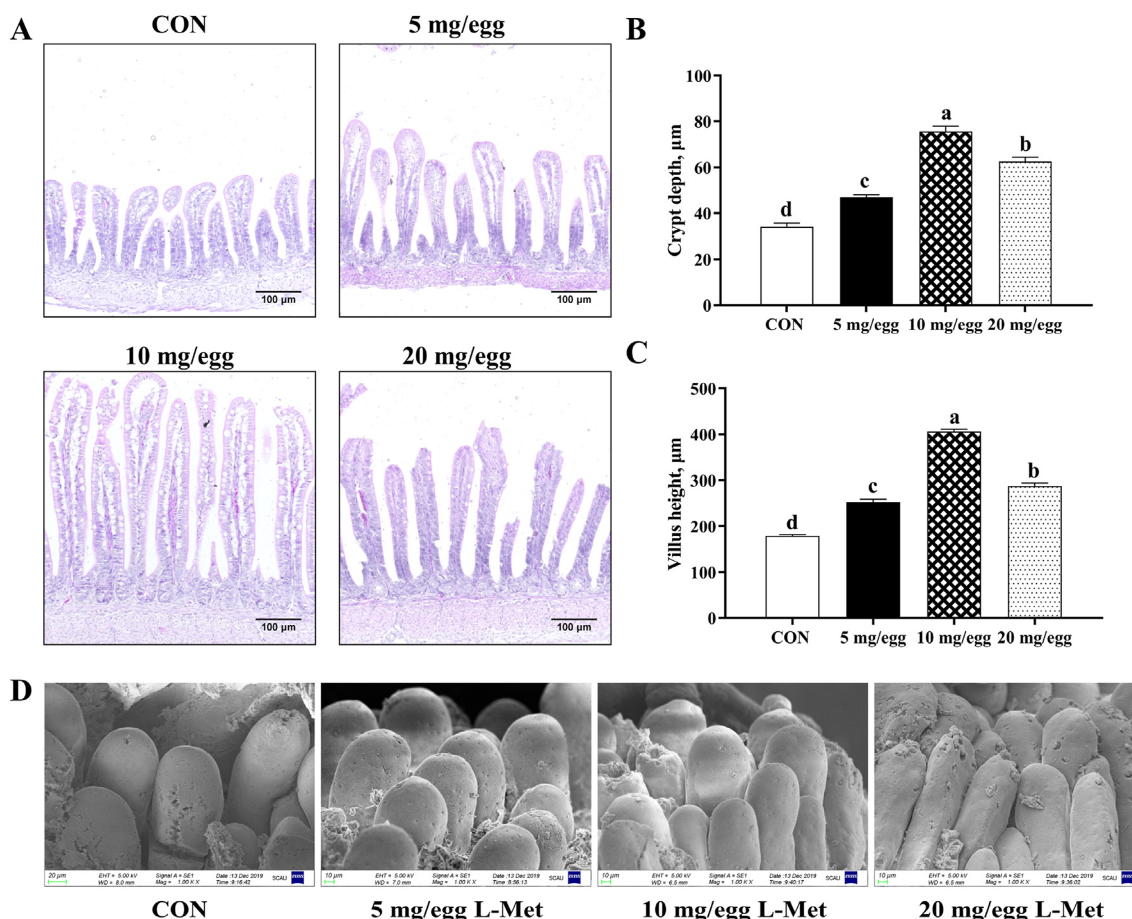

Fig. 2. The in ovo injection of methionine promotes intestinal epithelial integrity of chicks. (A) Representative images of H&E staining of the jejunum are shown (200 × magnification; Scale bars, 100 μm); (B to C) Statistical analysis of crypt depth and villus height; (D) Representative images of scanning electron micrographs of the jejunum (1,000× magnification). Bars without a common letter indicate significant differences ($P < 0.05$); the values are means ± SEM. $n = 6$. CON = control. L-Met = L-methionine.

processing. Serial sections of paraffin-embedded tissues with a thickness of 4 μm were cut using a microtome (Microm-HM340E, Thermo Fisher Scientific, Waltham, MA, USA) and stained with H&E. Then, images were captured under a microscope (Ti2–U, Nikon, Tokyo, Japan), and villus height and crypt depth were measured with Image-Pro Plus software.

2.4. Transepithelial electrical resistance (TEER)

To measure TEER, jejunum samples were balanced in Krebs–Ringer buffer (pH 7.4, 1.25 mmol/L NaCl, 2.5 mmol/L KCl, 1.25 mmol/L NaH_2PO_4, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 25 mmol/L $NaHCO_3$, 25 mmol/L glucose) for 10 min. Then, the samples were mounted directly onto and compressed between the two-chamber halves of an Ussing Chamber (Beijing Jingong Hongtai Technology CO., LTD., Beijing, China), representing the apical side and basolateral side, and surrounded by 7-mL Krebs–Ringer buffer on each side. The system was water-jacketed to 37 °C and carbonated with a carbogen (95% O_2 and 5% CO_2) gas flow. After an equilibration period of 30 min, the solutions were replaced with fresh Krebs–Ringer buffer, and then the experiments were run. Transepithelial electrical resistance (Ω/cm^2, resistance/surface area of the monolayer) was recorded as 3 consecutive measurements after subtracting the filter resistance value.

2.5. Scanning electron microscopy (SEM)

After fixing with 2.5% glutaraldehyde overnight, the jejuna were washed 3 times with PBS and treated with 1% osmium tetroxide for 1 h. Then, the jejuna were dehydrated with different concentrations of alcohol and dried to the critical point (EM CPD300, Leica Microsystems, Wetzlar, Germany), glued on stubs using conductive glue and coated with gold. Completely processed jejunum samples were examined using an EVO MA 15 SEM (Carl Zeiss, Jena, Germany).

2.6. Western blotting

Proteins were isolated from jejunum samples and analyzed as described previously (Xie et al., 2020). Total protein was extracted from the intestinal tissues using radioimmunoprecipitation assay lysis buffer, and the protein concentration in the homogenates was determined using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, Illinois, USA). The proteins and color prestained protein marker (M222-10, GenStar, Beijing, China) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, Massachusetts, USA). The band densities were quantified using ImageJ

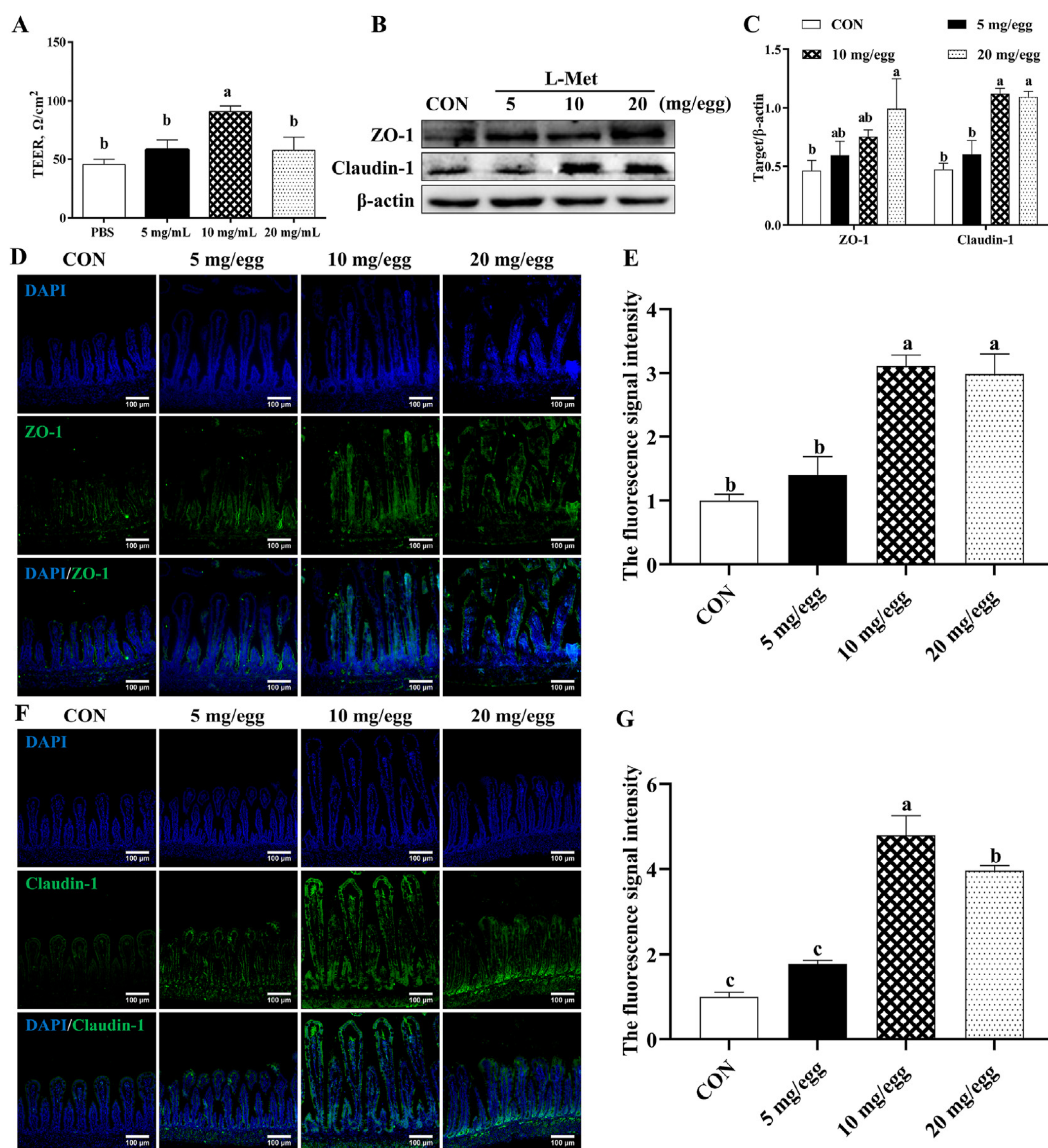

Fig. 3. The in ovo injection of methionine enhances the intestinal barrier function of chicks. (A) Transepithelial cell resistance (TEER) of the jejuna of chicks injected with phosphate-buffered saline (PBS) or L-methionine (L-Met). (B to C) The expression of ZO-1 and claudin-1 in the jejunum of chicks injected with PBS or L-Met. (D) Representative images of immunofluorescence (IF) staining with a ZO-1 antibody in the chick jejunum are shown (200× magnification; scale bar, 100 μm). (E) Statistical analysis of the fluorescence signal intensity based on the images shown in (D). (F) Representative images of IF staining with a claudin 1 antibody in the chick jejunum are shown (200× magnification; scale bar, 100 μm). (G) Statistical analysis of the fluorescence signal intensity based on the images shown in (F). Bars without a common letter indicate significant differences ($P < 0.05$); the values are means ± SEM. $n = 3$. CON = control.

software (version 1.8.0, National Institute of Health, Bethesda, MD, USA). The results were presented as the ratios of target protein level to β-actin level or the total protein level. The following antibodies were used: anti-p-JAK2 (#3771, CST, Cell Signaling Technology, Beverly, MA, USA), anti-JAK2 (#3230, CST), anti-p-STAT3 (#SC-8059, Santa Cruz Biotechnology, Dallas, TX, USA), anti-STAT3 (#SC-8019), anti-ZO-1 (#339100, Thermo Fisher), anti-Claudin-1 (#374900, Thermo Fisher), and anti-β-actin (#250132, ZENBIO, Chengdu, Sichuan, China).

2.7. Immunofluorescence

Immunofluorescence analysis was performed as described previously (Chen et al., 2020b). Fluorescence images were obtained with a microscope (NIS-Elements, Nikon, Tokyo, Japan). The following antibodies and reagents were used: anti-ZO-1, anti-claudin-1, and anti-Ki67 (#NB500-170, Novus Biologicals, Littleton, CO, USA), anti-Villin (#SC-58897, Santa Cruz Biotechnology); anti-p-JAK2, anti-p-STAT3, and FITC-conjugated secondary antibody (#115-545-003, Jackson Laboratory, Jackson, MS, USA); Cy3-conjugated secondary antibody (#111-165-045, Jackson Laboratory); and 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich).

2.8. Statistics

The data were analyzed using SPSS software version 20 (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was used to assess the differences between the 4 groups. *P*-values < 0.05 were considered statistically significant.

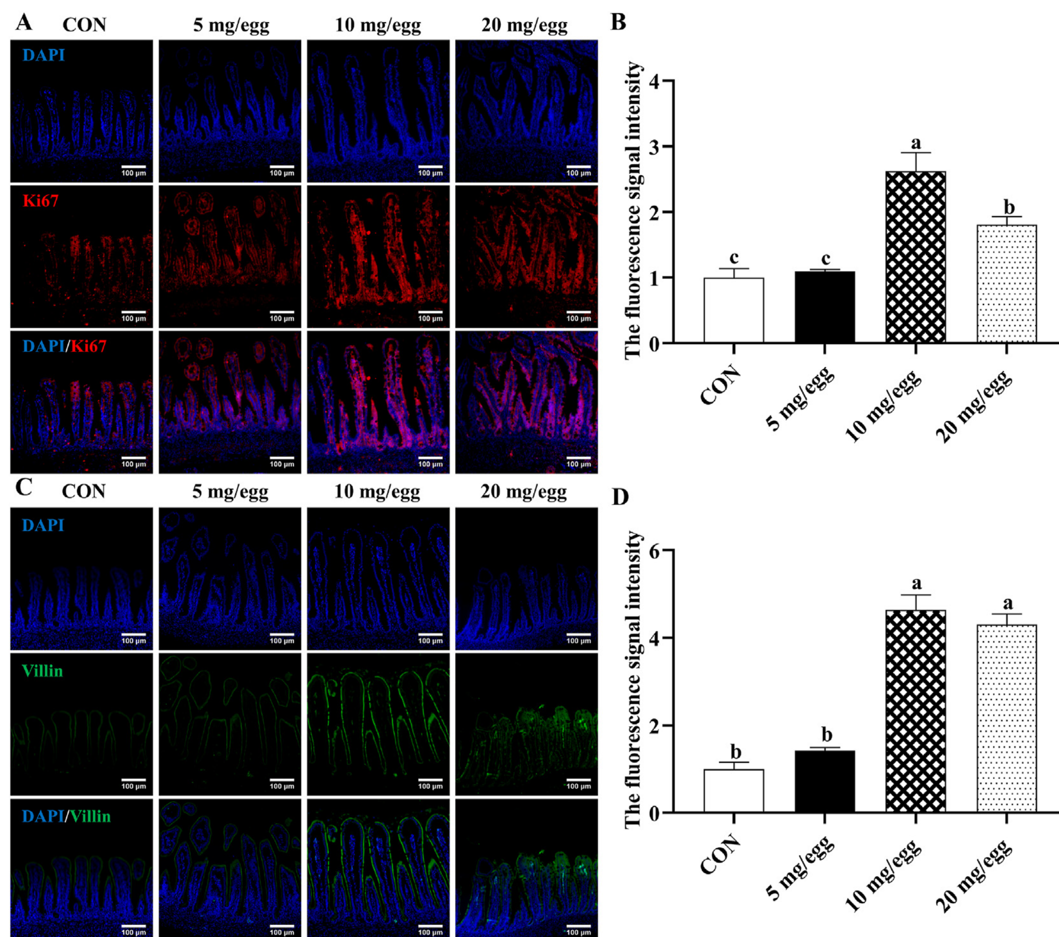

Fig. 4. L-Met promotes the proliferation and differentiation of intestinal cells. (A) Representative images of immunofluorescence (IF) staining with a Ki67 antibody in the chick jejunum (200× magnification; scale bar, 100 μm). (B) Statistical analysis of the fluorescence signal intensity based on the images shown in Fig. 4A. (C) Representative images of IF staining with a villin antibody in the chick jejunum are shown (200× magnification; scale bar, 100 μm). (D) Statistical analysis of the fluorescence signal intensity based on the images shown in Fig. 4 C. Bars without a common letter indicate significant differences ($P < 0.05$); the values are means ± SEM. $n = 3$. CON = control.

3. Results

3.1. L-Met promotes intestinal development and epithelial integrity

To study the effect of in ovo L-Met injection on the intestinal development of chick embryos, we measured the weight of the duodenum, jejunum, and ileum on the day of hatching and examined the morphology of the villi by H&E staining and SEM. The results showed that compared with the CON group, the chicks injected with L-Met did not have significantly altered body weight or small intestine length ($P > 0.05$, Fig. 1A and B). In particular, compared with the CON group, in ovo injection of 10 and 20 mg L-Met significantly increased the duodenal weight ($P < 0.05$, Fig. 1C). The 3 doses of L-Met markedly increased the jejunal weight ($P < 0.05$, Fig. 1C). For the ileum, 20 mg of L-Met injection significantly increased its weight ($P < 0.05$, Fig. 1C). H&E staining showed that the injection of 5, 10, and 20 mg of L-Met into the yolk significantly increased the villus height and crypt depth ($P < 0.05$, Fig. 2A, B and C). Further, the structural integrity of villi in the jejunum in the 5 and 10 mg of L-Met groups was better than that in the other groups (Fig. 2D).

3.2. L-Met enhances intestinal barrier function

To examine whether injection of L-Met into eggs can enhance the intestinal barrier function in the chick embryo, the TEER values and the tight junction protein expression of ZO-1 and claudin1 were determined. As shown in Fig. 3A, compared with the CON group, the group injected with 10 mg of L-Met showed significantly increased TEER values ($P < 0.05$), and the group injected with 20 mg of L-Met showed significantly increased expression of the ZO-1 and claudin-1 in the jejunum ($P < 0.05$, Fig. 3B and C). Similarly, the fluorescence signals of ZO-1 and claudin-1 on the crypt–villus axis were higher in all groups injected with L-Met than in the CON group. These data indicated that in ovo injection of L-Met enhances intestinal barrier function.

3.3. L-Met promotes the proliferation and differentiation of intestinal cells

Next, we tested the effect of L-Met on the proliferation and differentiation of intestinal epithelial cells. Higher fluorescence signal intensities of Ki67 (labels proliferating cells) ($P < 0.05$, Fig. 4A

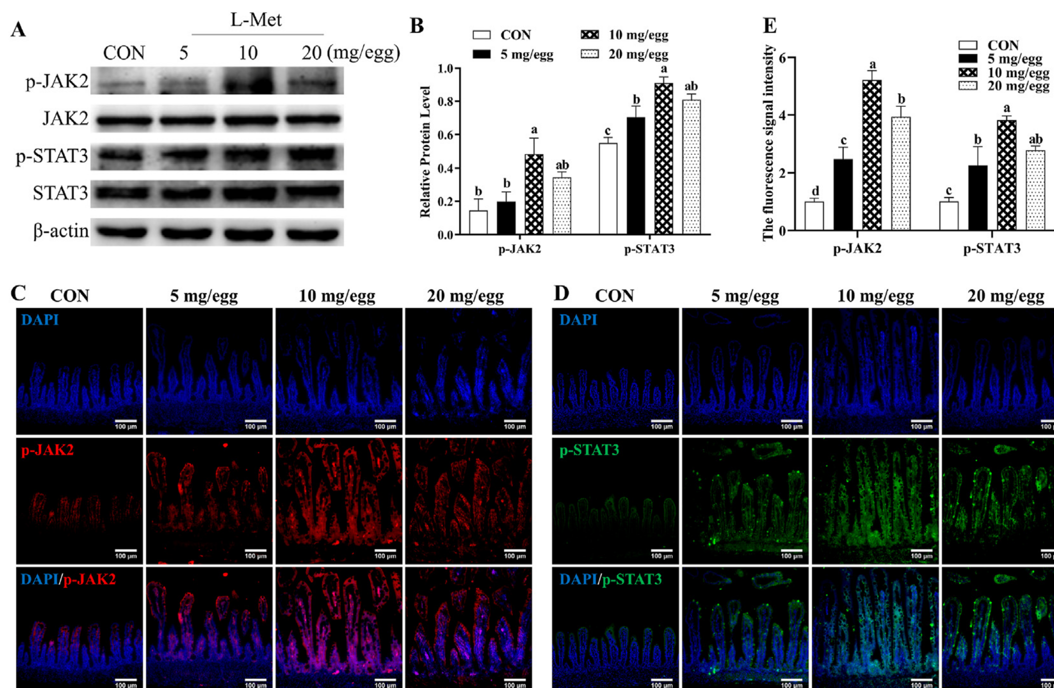

Fig. 5. The in ovo injection of methionine potentiates the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway activity in the jejunum of chicks. (A and B) The expression of p-JAK2 and p-STAT3 in the jejunum of chicks injected with phosphate-buffered saline (PBS) or L-methionine (L-Met). (C) Representative images of immunofluorescence (IF) staining with a p-JAK2 antibody in the chick jejunum are shown (200× magnification; scale bar, 100 μm). (D) Representative images of IF staining with a p-STAT3 antibody in the chick jejunum are shown (200× magnification; scale bar, 100 μm). (E) Statistical analysis of the fluorescence signal intensity based on the images shown in Fig. 5C and D. Bars without a common letter indicate significant differences ($P < 0.05$); the values are means ± SEM. $n = 3$. CON = control.

and B) and villin (labels absorptive cells) ($P < 0.05$, Fig. 4C and D) were observed after injecting L-Met. These results suggested that L-Met promotes ISC division and improves ISC differentiation into absorptive cells.

3.4. L-Met potentiates JAK2/STAT3 signaling pathway activity

Finally, we explored the possible mechanism of L-Met in intestinal development. Western blotting (Fig. 5A and B) and immunofluorescence (Fig. 5C, D, and E) showed that the protein expression of p-JAK2 and p-STAT3 was significantly increased in the L-Met group ($P < 0.05$). The results suggested that L-Met potentiates the JAK2/STAT3 signaling pathway.

4. Discussion

The present research demonstrates that supplementation with L-Met in ovo improves the intestinal structure and barrier function of chick embryos. Mechanistically, enterocytes respond to the nutritional environment (L-Met) to drive their rapid proliferation and differentiation, which promotes the renewal of the intestinal epithelium. As early as 1999, Ohta et al. (1999) found that the injection of amino acids into the yolk at E7 can accelerate the development of embryos without affecting their hatchability. Our recent investigation confirmed that 10 mg L-Met significantly increased the diameter and density of feather follicles (Chen et al., 2020a). Critically, evidence was presented that showed that the yolk membrane completely encloses the yolk at E9, indicating that the yolk injected with exogenous nutrients can be completely absorbed by the embryo body from this embryonic age (Chen et al., 2020a). The present data further support the beneficial effects of L-Met on chick embryos, including increased intestinal weight. Generally, the weight of the small intestine is considered an important factor that reflects the development of the small intestine. Zhou et al. (2020) demonstrated that deoxynivalenol causes a significant decrease in jejunum weight accompanied by severe villous atrophy and intensive crypt loss. In the present study, the significant rise in villus height and crypt depth after L-Met injection suggests increasing the absorptive surface area and is closely correlated to the increase in the mass and numbers of enterocytes, which was supported by villin and Ki67 staining.

An intact intestinal epithelial structure indicates a strong barrier function and is composed of monolayer intestinal epithelial cells and tight junctions between enterocytes. These tight junctions form a selective barrier that allows the transcellular transport of various dietary nutrients from the lumen into the internal circulation and restricts the passage of harmful substances (Chelakkot et al., 2018). The assembly and coordination of diverse tight junctions are essential for guarding the paracellular pathway. In particular, the transmembrane proteins claudins are linked to the actomyosin fibers of the cytoskeleton by Zonula occludens (Zos) (Wang et al., 2015). A previous study showed that Met deficiency impaired the intestinal function of chicks (Ruan et al., 2018). The supplementation of L-Met reverses the inhibitory effect of deoxynivalenol on the expression of ZO-1 and claudin-1 in the mouse intestine (Zhou et al., 2019). The difference is that we found that L-Met might enhance tight junctions in the embryonic jejunum in chicks under normal conditions. Collectively, these findings imply that L-Met may play a dual role in stimulating intestinal epithelial replenishment under homeostasis and regeneration after injury.

It is worth mentioning that the integrity of the intestinal structure and barrier function mostly depends on the proliferation and differentiation of ISC (Qi et al., 2020). These cells divide rapidly while migrating along the crypt–villus axis. Ki67 is indispensable for cells in the G1, S, G2, and M phases and determines cell proliferation activity. Our results proved that L-Met could maintain the rapid turnover of intestinal epithelium by accelerating cell proliferation in chick embryos. Subsequently, ISC differentiate into cells of various lineages in the villi, including absorptive cells, which account for more than 90% of all cells (Zhou et al., 2020). Villin is widely known as an F-actin bundling protein involved in the maintenance of the brush border of absorptive cells, and it is a sign of intestinal cell differentiation and maturation (Nick et al., 2015). We identified that Villin is highly expressed after L-Met supplementation, suggesting that L-Met could promote ISC differentiation and enhance nutrient absorption. Intestinal stem cell expansion is cross-controlled by a variety of signal transduction pathways in the ISC niche, including Wnt, Notch, JAK/STAT, and mTOR, which together ensure that the intestine is in long-term homeostasis. Richmond et al. (2018) demonstrated that ISC are initiated to proliferate and contribute to the subsequent regenerative response via the JAK/STAT signaling pathway. Furthermore, JAK/STAT3 activation strengthens intestinal epithelial barrier function (Kotelevets and Chastre, 2020; Zhao et al., 2020; Fang et al., 2019). Our present study indicated that L-Met could activate JAK2/STAT3 in the intestine of chick embryos; however, how to activate it is still unclear. There may be 3 ways to mediate this process, alone or in combination. The first is that L-Met enhances the assembly and secretion of cytokine ligands; the second is that L-Met directly binds to cytokine receptors; the third is that L-Met is transported to the cytoplasm and directly or indirectly modifies JAK2 itself or through its metabolites. The exploration of these problems will help to improve the signal network of embryonic intestinal development and advance the targeted regulation of functional amino acids.

In summary, through the chick embryo model, we found that in ovo injection of L-Met can enhance intestinal cell proliferation and differentiation to improve the structural and functional integrity of the intestinal epithelium through activation of the JAK2/STAT3 signaling pathway. Our results may provide new insights for regulating the intestinal development of embryonic chicks and the rapid growth of chicks after hatching.

Author contributions

Mengjie Chen: conceptualization, investigation, data curation, methodology, writing - original draft. **Jiayi Zhou**: methodology, data curation, writing - review & editing. Y**ijun Chen**: validation, methodology. **Xiuqi Wang**: supervision. **Huichao Yan**: resources. **Chunqi Gao**: conceptualization, funding acquisition, project administration, writing - review & editing.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

This work was jointly supported by the National Natural Science Foundation of China (31972585) and the Technical System of Poultry Industry of Guangdong Province, China (2021KJ128).

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