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Changes in chicken intestinal zinc exporter mRNA expression and small intestinal functionality following intra-amniotic zinc-methionine administration☆

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Abstract

A 303-bp cDNA of intestinal zinc exporter (ZnT1) was isolated from chicken jejunum by reverse transcriptase-polymerase chain reaction and sequenced, and showed 42% homology to *Homo sapiens* and *Rattus novergicus* intestinal ZnT1 genes. This specific probe was used to examine the effect of zinc-methionine (ZnMet) administration on the mRNA expression of ZnT1 and on small intestinal development and functionality. In this study, ZnMet was injected into the naturally consumed amniotic fluid of 17-day-old chicken embryos. The ZnT1 gene showed an approximately 200% increase in its mRNA levels from 48 h post-ZnMet injection, as compared to the control. An analysis of the gene expression of the brush-border enzymes and transporters showed increased mRNA expression of sucrase isomaltase, *leucine*-aminopeptidase, sodium-glucose cotransporter and Na^+K^+ATPase transporter (Na^+K^+ATPase) from 48 h post-ZnMet injection, in comparison to controls. Significant increases ($P<.05$) in the biochemical activity of the brush-border enzymes and transporters, and in jejunal villus surface area were detected from day of hatch (96 h post-ZnMet injection) as compared to controls. These results suggest that ZnMet administration into prenatal intestine via injection into the amniotic fluid enhances intestinal development and improves its functionality.

Keywords: Chicken; Embryo; Small intestine; Zinc-methionine; ZnT1 transporter

1. Introduction

Zinc plays an essential role in a wide variety of biochemical processes and is a required cofactor for the function of over 300 different enzymes [1,2]. Zinc participates in the synthesis of nucleic acids [3] and is an important structural cofactor for many proteins, including the ubiquitous zinc finger DNA-binding proteins [4].

The critical role of zinc in early embryonic development has been identified in studies indicating its presence in the embryo. Embryonic zinc deficiency induces proliferation arrest in many cells types [5], suppresses growth and causes congenital malformations that involve nearly all organ systems in offspring of zinc-deprived animals [6–8].

Zinc cannot cross biological membranes by simple diffusion since it is a highly charged, hydrophilic ion. Therefore, specialized mechanisms must exist for its cellular uptake and release. These uptake systems use integral membrane transport proteins to move zinc across the lipid bilayer of the plasma membrane.

Two families of zinc-transporter proteins, ZnT (zinc transporter) and ZIP [ZRT1 (zinc-regulated transporter), and IRT1 (iron-regulated transporter)-like proteins have been identified in mammals [9–13]. The ZnT proteins are members of the cation diffusion facilitator family and appear to function by either transporting zinc out of the cells or sequestering it in cellular compartments [10,12–15]. Seven ZnT proteins, ZnT1–ZnT7, have been identified and

Abbreviations: BBM, brush-border membrane; BW, body weight; LAP, *leucine*-aminopeptidase; Na^+K^+ATPase, Na^+K^+ATPase transporter; SI, sucrase isomaltase; SGLT-1, sodium-glucose cotransporter; ZnMet, zinc-methionine; ZnT1, zinc exporter.

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are predicted to have similar protein structures with features that include six transmembrane domains and a histidine-rich cytoplasmic loop between transmembrane domains IV and V [10]. ZnT1 was the first zinc transporter cloned [13] and it exhibits differential mRNA expression according to the amount of zinc in the diet [16,17]. Therefore, ZnT1 is responsive to zinc metabolism under specific conditions. Studies have shown that overexpression of ZnT1 in zinc-sensitive cells confers zinc resistance by direct export of unnecessary zinc out of the cells [2,18]. In addition, zinc supplementation leads to gene up-regulation for the normal processing function of aminopeptidase type 1 brush-border enzyme in vivo, suggesting that it may be important for this enzyme's proper functional alignment on the ribosomes [19].

The importance of zinc to intestinal development has been demonstrated in studies which showed increased intestinal crypt-cell production, reduced duration of mitosis in the distal intestinal segment [5] and improved epithelial cell restitution [20] in zinc-fed mice. According to these fundamental studies, we hypothesized that providing premature intestinal tissue with zinc would affect cellular and molecular processes in the intestine.

Zinc is less accessible in its mineral form [21,22]. Therefore, in this study, zinc was administered as zinc-methionine (ZnMet), an organically an available organic compound. ZnMet was injected into the consumed amniotic fluid of late-term chicken embryos, which are defined as having a premature intestine [23,24].

The current study examined the effect of ZnMet supplementation on the functional development of the small intestine, by measuring mRNA expression and biochemical activity of several brush-border membrane (BBM) enzymes and transporters, in addition to measuring the surface area of the intestinal villus. To confirm the intestinal tissue's response to zinc supplementation, a cDNA fragment of the chicken small intestine zinc transporter, ZnT1, was identified and its gene expression studied.

2. Materials and methods

2.1. Embryos and chicks

Embryos (Ross × Ross) were obtained from a commercial hatchery (Goldkist Hatchery, Siler City, NC), from a maternal flock 35 weeks in lay. The eggs were incubated under optimal conditions. On day 17 of incubation (day 17E), all eggs containing viable embryos received injections into the amnion. Upon hatching, 10 chicks per 1-m^2 pen were randomly assigned to treatment groups and housed in a total-confinement building. Each pen was equipped with an automatic nipple drinker and manual self-feeder. All birds were given ad libitum access to water and diet formulated to meet NRC recommendations [25]. Experimental protocols were approved by the Institutional Animal Care and Use Committee.

2.2. Tissue sampling

Ten embryos or chicks from each group (ZnMet and Control) were euthanized at 18, 19 and 20 days of incubation (days 18E, 19E and 20E, respectively), on day of hatch (within 2 h of clearing the shell), and at 7 days posthatch. The jejunum was removed as previously described [26,27] and 1-cm-long segments were placed in three separate tubes: (1) fixed in 4% neutral-buffered formalin solution for histology, (2) stored at −20°C for determination of BBM enzymes and transporters [sucrase-isomaltase (SI), *leucine*-aminopeptidase (LAP), alkaline phosphatase, and γ-glutamyltransferase (GGT) activities], (3) frozen in liquid nitrogen and stored at −80°C for RNA expression analysis.

2.3. Intra-amniotic administration

At 17 days of embryonic incubation (17E), eggs containing viable embryos were weighed and divided into two treatment groups of 100 eggs, each with an average egg weight of 55±1.45 g. All treatment groups were assigned eggs of similar weight-frequency distribution. Each group of 100 eggs was then injected with the specified solution (1 ml per egg) with a 21-gauge needle into the amniotic fluid, which was identified by candling [23]. The intra-amniotic treatment solutions included the following: (1) ZnMet— 0.5 g/L ZnMet, 5 g/L NaCl; (2) Control— −5 g/L NaCl. After all the eggs were injected, the injection holes were sealed with cellophane tape and the eggs placed in hatching baskets such that each treatment was equally represented at each incubator location. Hatchability was similar in treatment and control groups, approximately 90% (data not shown).

2.4. Isolation of a chicken intestinal ZnT1 gene fragment

Intestinal jejunal sections were homogenized in TRI-REAGENT [Molecular Research Center (MRC), Cincinnati, OH] to isolate total RNA and reverse-transcribed using oligo(dT) and Superscript II reverse transcriptase (MBI, Fermentas). Primers were designed to correspond to nucleotides 214–235 (5′-GCTGCTGCTGACCTTCATGTTC-3′) and 552–574 (5′-ACAGGCAGAGCCCCAGCACGTT-3′) of the previously published rat small intestinal ZnT1 sequence [16]. Polymerase chain reaction (PCR) was performed by using *Pfu* DNA polymerase (Promega, Madison, WI) in 40 amplification cycles at an annealing temperature of 55°C. The isolated PCR fragment of 303 bp was subjected to automated sequencing using an Applied Biosystem 373A DNA sequencer. Nucleic acid sequences were analyzed and homology between chicken and other ZnT1 sequences was calculated using DNAMAN version 4.0 (Lynnon Biosoft).

2.5. Total RNA extraction

Total RNA was isolated from the jejunal section using TRI-REAGENT (0.001 L/100 mg tissue) according to the manufacturer's protocol (MRC).

2.6. Northern blot hybridization

Six probes were used for the hybridization: (1) a 786-bp cDNA fragment of chicken SI [28]; (2) a 522-bp cDNA fragment of chicken LAP [29]; (3) a 970-bp cDNA fragment of chicken sodium-glucose cotransporter (SGLT-1) [30]; (4) a 1141-bp cDNA fragment of chicken Na^+K^+ATPase transporter (Na^+K^+ATPase) [31]; (5) the isolated 303-bp cDNA fragment of chicken ZnT1 (AJ619980) and (6) a 419-bp cDNA fragment of chicken 18S rRNA [23], which was used to normalize variations in total RNA loading. For Northern blot analysis, 30 μg of total RNA was denatured and separated by electrophoresis on a 1.5% agarose–1.1 mol/L formaldehyde gel. After electrophoresis, RNA was transferred overnight by capillary transfer onto a positively charged nylon Hybond-N membrane (Amersham Pharmacia Biotech, Bucks, UK) and then cross-linked by UV radiation at 340 nm for 2 min. The probes were labeled with ^{32}P-dCTP by random priming (Biological Industries). Prehybridization was at 42°C for 4 h, hybridization was conducted at 42°C overnight, and a high-stringency wash [saline sodium citrate/0.1% SDS at 50°C] was performed according to the procedures recommended by Amersham for Hybond-N membranes. Blots were exposed for 17–48 h at −80°C to Kodak XAR 5 film with an intensifying screen. The abundance of BBM nutrient-transporter and digestive-enzyme transcripts was normalized to the density of the 18S rRNA transcripts by densitometry with a high-resolution scanner. Gel-Pro densitometer software (Version 3.0, Media Cybernetics, Silver Spring, MD) was applied to determine the amount of mRNA in each band. The expression of mRNA is presented in arbitrary units.

2.7. Brush-border enzyme activity

Enzyme activities were assayed using jejunal lysates (250 mg tissue/5 ml of 50 mM sodium phosphate buffer, pH 7.2). Maltase (EC 3.2.1.20) activity was assayed colorimetrically using maltose as a substrate [32–34] and expressed as millimoles of glucose released per minute per gram of jejunal protein. LAP activity (EC 3.4.11.2) was determined by hydrolysis of *l*-leucine-*p*-nitroanilide for 15 min at 37°C, and *p*-nitroanilide was determined spectrophotometrically at 405 nm according to Benajiba and Maroux [35]. One unit of LAP activity was defined as the production of 1 μmol *p*-nitroanilide per minute per gram of jejunal protein. Total protein was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

2.8. Morphological examination

Intestinal samples from days 17E, 18E, 19E, day of hatch and 7 days posthatch from each treatment were dehydrated, cleared and embedded in paraffin. Serial sections (5 μm) were placed on glass slides, deparaffinized in xylene, rehydrated in a graded alcohol series and examined by light microscopy.

Morphometric measurements were performed with an Olympus light microscope using EPIX XCAP software (EPIX, Buffalo Grove, IL). Villus surface area was calculated from villus height and width at half height [28].

2.9. Statistical analysis

Results were analyzed by ANOVA using the general linear models procedure of SAS software [36]. Differences between treatments were compared by Tukey's test and values were considered statistically different at $P<.050$ unless otherwise stated.

3. Results

3.1. Isolation and sequencing of partial chicken intestinal ZnT1 cDNA

A 303-bp fragment of the chicken intestinal ZnT1 gene was isolated by reverse transcriptase-PCR and subjected to sequence analysis. It exhibited 42% homology to *Homo sapiens* and to *Rattus novergicus* intestinal ZnT1 genes

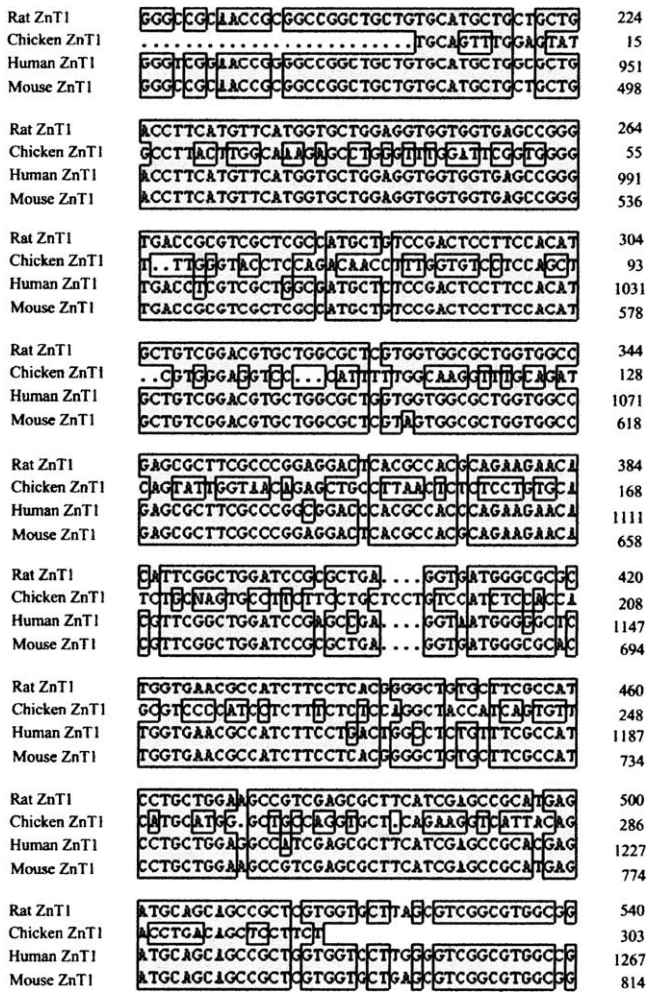


Fig. 1. Predicted partial amino acid sequences of the chicken intestinal ZnT1 exporter. The alignment of predicted amino acid sequences of chicken intestinal ZnT1 with human ZnT1 (GI12382778), mouse ZnT1 (GI577840) and rat ZnT1 (GI577842) is shown. Homologous residues are shaded.

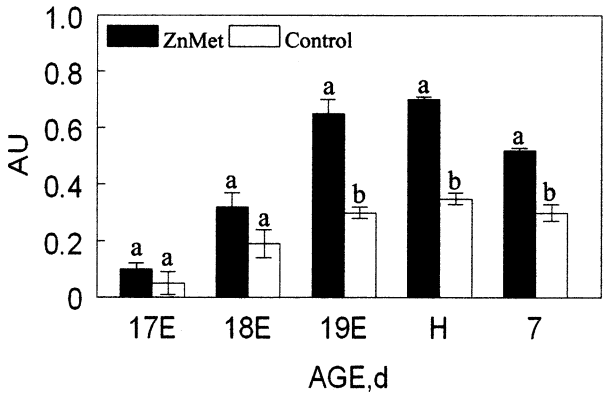


Fig. 2. Chicken jejunum intestinal mRNA expression in control and intra-amniotically administered ZnMet embryos and chicks. Changes in mRNA expression were analyzed by Northern blot analysis for ZnT1 from embryonic and posthatch chick jejunums on days 17E, 18E and 19E, day of hatch and 7 days posthatch. Values are means±S.E.M., $n=4$. Means with different letters differ at $P<.05$.

(Fig. 1). The cDNA sequence of the chicken intestinal ZnT1 was entered into the EMBL nucleotide sequence database under accession number AJ619980. The amino acid sequence of this fragment resulted in a predicted translation product of 101 amino acids. This amino acid sequence was 32% homologous to *H. sapiens* intestinal ZnT1, and 30% homologous to *R. novergicus* intestinal ZnT1. A search of the conserved domain database revealed that the partial sequence is located between the second and third cross-membrane domains of ZnT1.

3.2. mRNA expression of intestinal ZnT1

A 60% increase in ZnT1 mRNA level ($P<.05$) was observed in ZnMet birds 24 h post-ZnMet administration. A 200% increase in the gene's expression was observed 48 and 96 h postinjection, whereas at 7 days posthatch, its level was 50% higher than in control birds (Fig. 2).

3.3. mRNA expression of BBM enzymes and transporters

Northern blot analysis, followed by quantity determination relative to 18S rRNA, revealed increased mRNA expression of SI and LAP enzymes in the ZnMet birds 24 h post-ZnMet administration, relative to the control group (Fig. 3). Sucrase isomaltase expression was elevated by 25% on day of hatch and by 80% 7 days posthatch. No

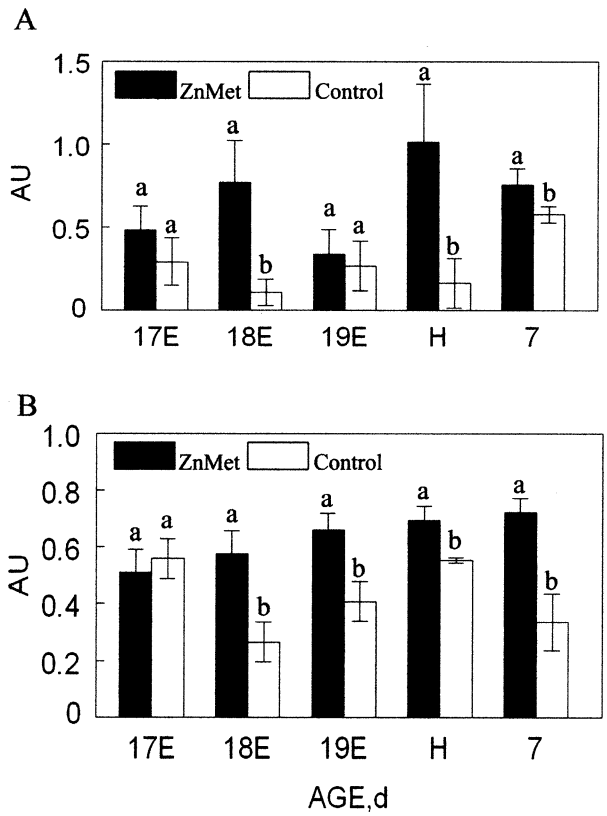


Fig. 3. Chicken jejunum intestinal mRNA expression in control and intra-amniotically administered ZnMet embryos and chicks. Changes in mRNA expression were measured by Northern blot analysis for SI (A) and LAP (B), from embryonic and posthatch chick jejunums on days 17E, 18E and 19E, day of hatch and 7 days posthatch. Values are means±S.E.M., $n=4$. Means with different letters differ at $P<.05$.

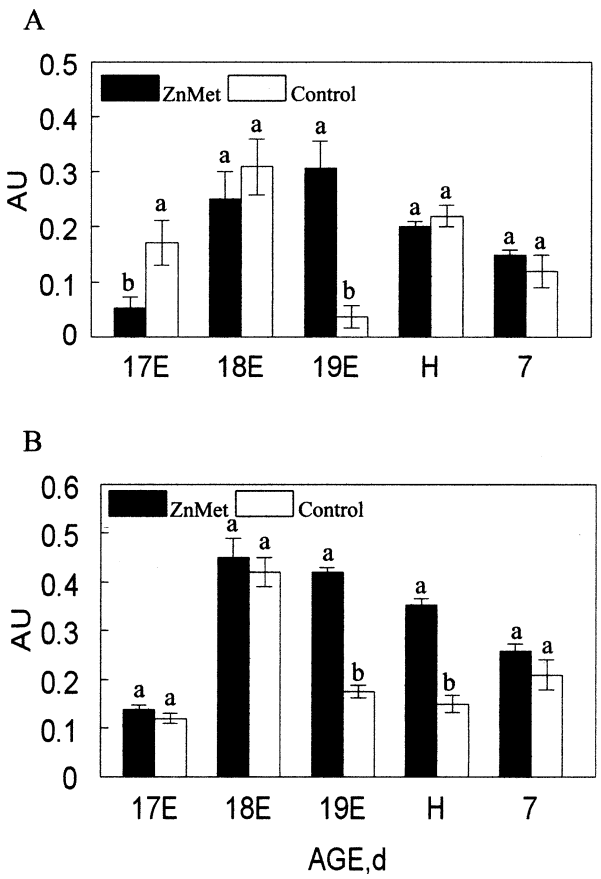
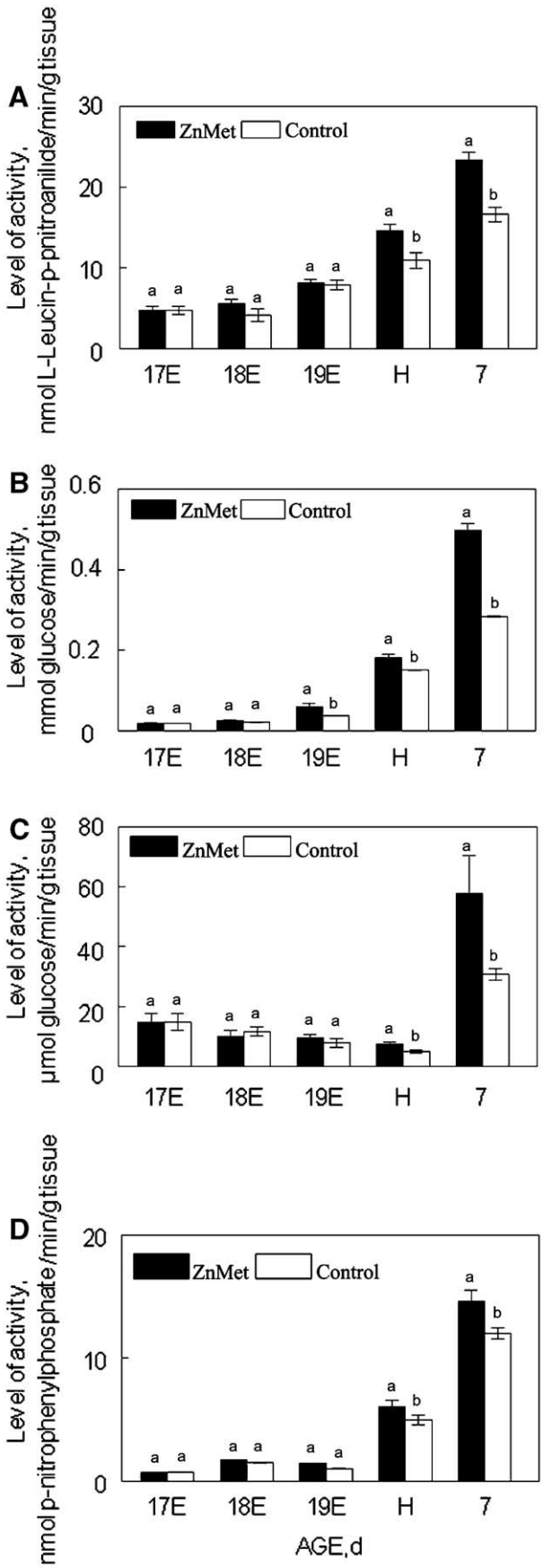


Fig. 4. Chicken jejunum intestinal mRNA expression in control and intra-amniotically administered ZnMet embryos and chicks. Changes in mRNA expression were measured by Northern blot analysis for SGLT-1 (A) and Na^+K^+ATPase (B) from embryonic and posthatch chick jejunums on days 17E, 18E and 19E, day of hatch and 7 days posthatch. Values are means ±S.E.M., $n=4$. Means with different letters differ at $P<.05$.



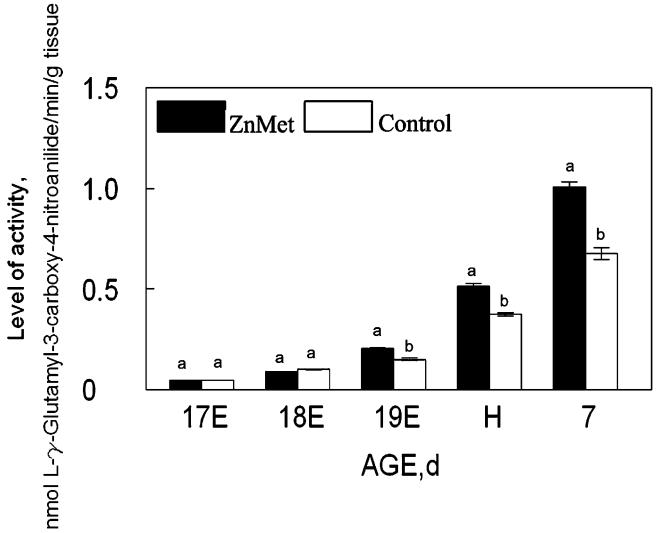


Fig. 6. Biochemical activity of the brush-border transporter GGT in control and ZnMet birds on days 17E, 18E and 19E, day of hatch and 7 days posthatch. Values are means±S.E.M., $n=10$. Means with different letters differ at $P<.05$.

significant difference was observed on day 19E. However, LAP expression was consistently higher (by 15–45%) in the ZnMet group compared to controls.

SGLT-1 and Na^+K^+ATPase expression was higher by 50% and 25%, respectively, 48 h post-ZnMet administration compared to controls (Fig. 4). This pattern of gene expression was only maintained on day of hatch for Na^+K^+ATPase, whereas no differences in the expression of these transporters were detectable by day 7.

3.4. Biochemical activity of the jejunal BBM enzymes and transporter

LAP revealed higher levels of biochemical activity ($P<.05$) in the ZnMet birds, from day of hatch (96 h post-ZnMet administration) to 7 days posthatch, relative to controls. In general, LAP activity increased gradually from day 17E until day 7 in all groups (Fig. 5A).

Sucrase isomaltase showed a similar pattern of biochemical activity (Fig. 5B and C) in ZnMet and control birds, whereas alkaline phosphatase revealed higher levels of activity ($P<.05$) on day 7 in the amniotic administrated birds (Fig. 5D). The biochemical activity of GGT was significantly higher ($P<.05$) in the ZnMet vs. control birds from 48 h post-ZnMet administration (Fig. 6).

3.5. Morphological measurements

Histological measurements indicated that 48 and 96 h post-ZnMet administration and 7 days posthatch, the small intestine of the ZnMet birds had a significantly larger

Fig. 5. Biochemical activity of the brush-border enzymes LAP (A) sucrase (B), maltase (C) and alkaline phosphatase (D) in control and ZnMet birds on days 17E, 18E and 19E, day of hatch and 7 days posthatch. Values are means±S.E.M., $n=10$. Means with different letters differ at $P<.05$.

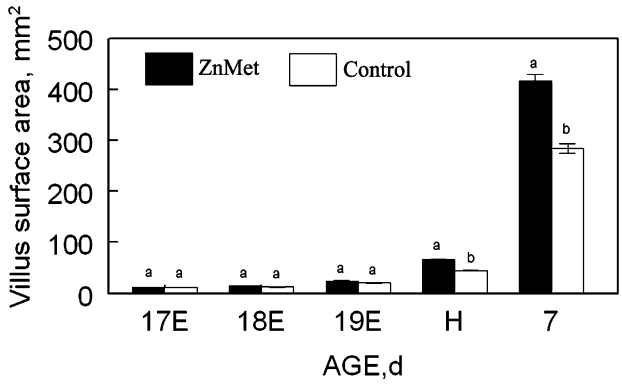


Fig. 7. Effect of intra-amniotic administration of ZnMet on days 17E, 18E and 19E, day of hatch and 7 days posthatch on the jejunum small intestinal villus surface area. Values are means±S.E.M., $n=4$. Means with different letters differ ($P<.05$).

villus surface area ($P<.05$), by 15%, 25% and 47%, respectively (Fig. 7).

4. Discussion

This study showed that ZnMet administration to the developing intestinal tissue leads to gene up-regulation and increased biochemical activity of the BBM transporters and enzymes, as well as a significant increase in intestinal villus surface area.

Cells in general, and enterocytes in particular, maintain zinc concentrations within relatively narrow limits [37,38], because high cellular zinc concentrations are toxic [5,37]. It appears that changes in free zinc pools or in zinc bound to specific ligands or within intracellular vesicles may occur without a major change in total cellular zinc [22]. The ZnT1 exporter, located on the basolateral side of the cell, has been identified as a major rescue agent for cells maintained in high extracellular zinc concentrations [12,13]. ZnT1 regulation by dietary zinc has been shown in rats, whereby 7 days of feed supplementation with 180 mg Zn/kg led to greater zinc intake due to the higher mRNA expression and activity of the ZnT1 exporter relative to controls [16].

In this study, the increased expression of ZnT1 from 48 h post-ZnMet administration indicated that intestinal enterocytes regulate zinc levels by exporting the redundant cellular zinc via the ZnT1 exporter. Consequently, it can be concluded that in our procedure, intra-amniotically administered zinc can cross the intestinal luminal membrane into the enterocyte. Therefore, ZnT1 can serve as an indicator of the developing intestinal tissue's ability to respond to dietary zinc. We did not study other zinc transporters here because previous studies [12,13,22] have shown ZnT1 to be a reliable indicator.

We isolated and sequenced a fragment of the chicken intestinal ZnT1 gene. The predicted amino acid sequence of the isolated gene fragment showed similar homology (42%) to both humans and rats [13]. The differences between chicken intestinal ZnT1 and mammalian ZnT1 genes might be due to evolutionary divergence. However, despite the differences in homology, the chicken ZnT1 protein included the predicted conserved domain located between the second and third, out of a possible six putative, membrane-spanning domains of the ZnT1 transporter [12,13].

Since zinc is less available in its mineral form, ZnMet, an organic, available zinc compound was used. This choice was based on previous studies [21,39] which showed that rats receiving methionine supplement absorb 82% of the dietary zinc provided vs. 64% in controls. Although there are other ways of providing zinc supplementation [5,40], the advantage of including ZnMet in the diet has been demonstrated in previous studies. Experiments have shown that feed containing ZnMet results in a positive wool-growth response in sheep and an increase in lambs' daily weight gain ($P<.05$) [41]. In addition, steers fed ZnMet had better carcass quality and greater marbling scores than controls [42]. Further experiments revealed that the number of red blood cells ingested per phagocytic cell is increased ($P<.05$) following 80 ppm ZnMet supplementation in weanling pig feed [43]. Supplemental ZnMet in poultry diets increased cellular immune response in progeny as well as embryonic bone weight in both chicken and mouse [40,44]. In addition, previous studies have shown that exogenously administered zinc enhances intestinal repair of the epithelium via the stimulation of some relevant mechanisms, and that zinc may promote intestinal epithelial wound healing by enhancing epithelial cell restitution, the initial step in this healing process [38,45]. The research presented here concurs with these previous studies, since intra-amniotic supplementation of ZnMet led to increased intestinal villus development, as well as BBM enzyme and transporter gene expression and biochemical activity.

Although the effect of ZnMet supplementation has been previously examined and the role of zinc in aminopeptidase N regulation demonstrated [46,47], its effect on the intestinal BBM was never investigated in birds. Based on the knowledge that aminopeptidase N is a zinc-dependent enzyme [29,48,49] and that its zinc finger domain is essential for the normal processing functions of the aminopeptidase enzyme family [50,51], we demonstrated in this study that the administration of zinc into the amniotic fluid increases the BBM enzymes mRNA expression, resulting in increased BBM enzymatic activity.

Zinc participates in the regulation of cell division, proliferation [5,45], DNA synthesis and mitosis [45,52], and is a structural constituent of a great number of proteins, including enzymes of cellular signaling pathways and transcription factors [2,7]. Therefore, it is reasonable to conclude that zinc might be responsible for the enlargement of the intestinal villus surface area and the increased functionality of all BBM enzymes and transporters studied here in the ZnMet birds relative to controls.

Numerous studies support the importance of methionine in nuclear processes such as protein synthesis [53–56].

However, in this study, the intestinal functional and developmental alterations are not necessarily related to either zinc or methionine separately, but to their combination.

Thus, it appears that the administration of dietary ZnMet into the chick embryo's amniotic fluid triggers increased expression of the genes involved in the digestion and uptake of amino acids and glucose. This results in enhanced capacity of the BBM transporters and enzymes and consequently, intestinal development.

This study also introduces an innovative approach for presenting exogenous nutrients to developing tissue. Intra-amniotic administration into the late-term embryo may prove useful in investigating the effects of specific nutrients at particular stages of intestinal development.

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