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Effects of Zinc on Cell Proliferation, Zinc Transport, and Calcium Deposition in Primary Endometrial Epithelial Cells of Laying Hens In Vitro

Lanlan Li[1,2] · Wenting Zhou[1] · Sasa Miao[1] · Xinyang Dong[1] · Xiaoting Zou[1]

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Abstract

For birds, the uterus is an important part for eggshell mineralization, and the establishment of the endometrial epithelial cell (EEC) model was beneficial to the study of uterine function. This study was conducted to establish a culture model of primary EECs of laying hens and explore the effects of zinc on primary EEC proliferation, zinc transport, and calcium deposition in vitro. The EECs were isolated and cultured via type I collagenase digestion, and in the logarithmic phase during 2–5 days, and then reached the plateau phase on the 7th day of inoculation. Results showed that the proliferation of EECs treated by 50 μM $ZnSO_4$ or zinc-methionine (Zn-Met) were markedly promoted at 24-h or 48-h treating time ($P < 0.05$). In later experiments, the EECs were divided into three groups, involving a control group (no zinc treated), $ZnSO_4$ group (50 μM zinc treated) and a Zn-Met group (50 μM zinc treated). Results showed the relative fluorescence intensity of Ca^{2+} in the Zn-Met group was significantly higher than that in the control group ($P < 0.05$). As for zinc transporters, it was only observed that mRNA levels of metallothionein (MT) in EECs showed a significant difference ($P < 0.05$) between the Zn-Met group and the control. In conclusion, the EECs of laying hens isolated by scraping and digested collagenase I were with better adherent growth. Moreover, Zn-Met can increase intracellular Ca^{2+} concentration and upregulate expressions of MT mRNA in the EECs of laying hens.

Keywords Calcium deposition · Endometrial epithelial cell · Laying hen · Zinc transporter · Zn-methionine

Abbreviations

CA	Carbonic anhydrase
$CaBP\text{-}D_{28k}$	Ca-binding protein-d_{28k}
CLU	Clusterin
DMT1	Divalent metal transport 1
EECs	Endometrial epithelial cells
MT	Metallothionein
MTF-1	Metal-response element-binding transcription factor-1
OC-116	Ovocleidin-116
OC-17	Ovocleidin-17
OVA	Ovalbumin
OVOT	Ovotransferrin
OPN	Osteopontin
PBS	Phosphate-buffered saline
ZIP5	Zrt- and Irt-like protein 5, SLC39A5
Zn-Met	Zinc-methionine
ZnT1	Zinc transporter 1, SLC30A1
ZnT5	Zinc transporter 5, SLC30A5

✉ Xiaoting Zou
xtzou@zju.edu.cn

[1] Key Laboratory of Molecular Animal Nutrition, Ministry of Education, Feed Science Institute, College of Animal Science, Zhejiang University, Hangzhou 310058, People's Republic of China

[2] Hubei Key Laboratory of Animal Nutrition and Feed Science, Hubei Collaborative Innovation Center for Animal Nutrition and Feed Safety, Wuhan Polytechnic University, Wuhan 430023, People's Republic of China

Introduction

Avian eggshell is a complex biomaterial made of 95% calcium carbonate in its calcitic form and 3.5% organic matrix component, which is a complex mixture of proteins,

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glycoproteins, and proteoglycans [1–5]. Uterus, as the part that forms the eggshell, daily secretes organic and mineral precursors required for eggshell mineralization during a period of about 20 h into a cell-free medium (uterine fluid), which bathes the egg during three phases of shell mineralization [6]. It was known that eggshell formation requires large amounts of ionic precursors for $CaCO_3$ [7]. However, both elements (Ca^{2+} and HCO_3^-) are not stored in the uterus but are continuously supplied during eggshell formation by the blood plasma via trans-epithelial transport taking place across the uterine glandular cells [8–10]. Moreover, the endometrium consists of a single layer of epithelium plus the stroma on which the epithelium rests [11]. Up to now, many reports on the uterine epithelium cells have been made in a variety of mammals, including rats, pigs, rabbits, monkeys, and sheep [12–16]. However, the establishment of a cultivation system of primary endometrial epithelial cells (EECs) of laying hens in vitro was studied in few reports.

In addition, trace minerals are essential in the diet of laying hens because they participate in biochemical processes necessary for normal growth and development, including bone and eggshell formation [17, 18]. Zinc is necessary for the maintenance of structural and functional integrity of biological membranes, facilitation of gene expression and protein synthesis, enzyme structure and function, and appetite regulation [19, 20]. Moreover, zinc is a co-factor of the enzyme carbonic anhydrase (CA) which involves catalyzing hydration of metabolic CO_2 to HCO_3^- and supplying carbonate ions during eggshell formation [21]. In our previous study, it was found that the increase in zinc-methionine (Zn-Met)-induced Ca deposition may be due to increased zinc contents in the serum and tissues, which were attributable to the increased CA concentrations in the serum, increased Ca and albumin levels, and upregulated CA and Ca-binding protein-d_{28k} ($CaBP\text{-}D_{28k}$) mRNA levels in the eggshell gland (ESG) [22]. However, effects of zinc on calcium deposition-related parameters in vitro were not studied.

Therefore, the objective of the present study was to establish a culture model of primary EECs of laying hens and explore effects of zinc on cell proliferation, zinc transport, and calcium deposition in primary EECs in vitro. In the present study, effects of zinc on intracellular CA activity, calcium ion in cell and culture medium, and expressions of genes involved in zinc transfer and calcium deposition in the EEC model of laying hens were investigated.

Material and Methods

All experiments performed on animals were conducted in accordance with the Animal Welfare Committee guidelines and approved by the Animal Science College of Zhejiang University (Hangzhou, China).

Experimental Animals

Hyline gray layers (32 weeks) randomly chosen as the experimental animals were in good physical condition without uterine disease and at the peak of egg production (laying rate: ≧ 85%). An enclosed, ventilated, and conventional house with 16 h of lighting was provided for all laying hens to keep them in the same environmental conditions. Feed and water were offered ad libitum.

Separation and Cultivation of Endometrial Epithelial Cells

Firstly, laying hens were sacrificed humanely by carbon dioxide asphyxiation and the uterus was collected immediately with two head ligations and put in phosphate-buffered saline (PBS, Biosharp). The connective tissue, blood vessels, and non-correlated tissue (vagina) were all removed from the uterus under sterile PBS. In the following, the uterus was cut open with sterile scissors and washed three times with sterile PBS. And then the endometrium was scraped with a scalpel and the scraped tissues were suspended in Dulbecco's Modified Eagle's Medium/nutrient mixture F-12 (DMEM/F12, HyClone Corporation, USA). After repeated pipette blowing, the mixture was centrifuged for 5 min at 1500 rpm and the sediment was digested by immersion in a solution containing type I collagenase (1 mg/mL, Sigma Aldrich) at 37 °C for 60 min. The digestion was terminated using a culture medium containing 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biotechnology Materials Limited Company), and the mixture was filtered using three layers of sterile gauze and then the filtrate was centrifuged for 5 min at 1500 rpm. The sediment was washed twice and then suspended in a complete culture medium (DMEM/F12 supplemented with 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Biological Industries)). And finally, the cell viability was determined to be above 90% by the trypan blue exclusion method. Cell density was adjusted to 10^6 cells/mL, then the cells were seeded into 6-well plates and 96-well plates (Corning) at 37 °C in air containing 5% CO_2, respectively.

Analysis for Growth Curve of Epithelial Cells

The epithelial cells were cultured in 96-well plates at a density of 1×10^5 cells/well in complete culture medium (0.1 mL) at 37 °C in air containing 5% CO_2 (changing the medium per 24 h). A total of 7 groups were set up (4 replications/group, 8 wells/replication). The cell growth curves of epithelial cells were determined by using the CCK8 (Vazyme) assays. After incubation for 24 h, 10 μl CCK8 solution was individually added into 8-parallel wells, which were then covered with a sheet of tinfoil and cultured for an additional 1.5 h at 37 °C in 5% CO_2/air. The 96-well plate was read on a microplate

reader (Biotek ELX800, Biotek Instruments, Inc., Winooski, USA) at 450 nm. The same observations lasted 7 days.

Effects of Zinc on the Proliferation of Epithelial Cells In Vitro

The epithelial cells were cultured in 96-well plates at a density of 1×10^5 cells/well in complete culture medium (0.1 mL) at 37 °C in air containing 5% CO_2 (changing the medium per 24 h). The experiments were divided into three groups, including the control group treated by PBS, the zinc sulfate ($ZnSO_4$, ≧ 99.00% in purity, Sinopharm Chemical Reagent Co. Ltd.)-treated group, and the Zn-Met (rate of chelation: 99.0%, purity: 96.00%, 16.00% of zinc content, 80.00% of methionine, Novus International Trading Co., Ltd., Shanghai)-treated group, as shown in Table 1. On the third day of cultivation, different concentration gradients and different forms of zinc (dissolved in sterile PBS) were added to the medium, and effects of zinc on the proliferation of epithelial cells were determined by using the CCK8 assays mentioned above at 24-h and 48-h zinc treatment time, respectively. Experiments were conducted with replicates of 8-parallel wells per treatment condition. From these experiments, the optimal zinc concentration and treatment time could be obtained for future experiments.

Determination of Intracellular Carbonic Anhydrase Activity

The epithelial cells isolated were seeded into 6-well plates at a density of 5×10^5 cells/well in complete culture medium (2.5 mL) and divided into 3 groups (6 wells/group), involving the control group, $ZnSO_4$ group, and Zn-Met group. Cells were maintained in a humidified environment with an atmosphere of 5% CO_2 at 37 °C. On the third day of cultivation, different forms of zinc were added to the medium according to the above experiment.

The epithelial cells were washed three times by sterile PBS and treated by ultrasonication, then centrifuged for 5 min at 5000 rpm. The supernatant collected was used to determine cellular CA activity using ELISA kits (Shanghai Mlbio Institute, Shanghai, China). A microplate reader (Biotek ELX800, Biotek Instruments, Inc., Winooski, USA) at 450 nm was used in the determination.

Table 1 Different zinc experimental treatments on epithelial cells of laying hens in vitro

	$ZnSO_4$ (μM)	Zn-Met (μM)
Control group	–	–
$ZnSO_4$ group	25	–
	50	–
	100	–
	200	–
	400	–
Zn-Met group	–	25
	–	50
	–	100
	–	200
	–	400

The horizontal line (–) represents those not added zinc sources, such as Zn-Met or $ZnSO_4$. The control group were added sterile PBS without zinc, and each zinc treatment group consisted of five different treatments that were added with different concentration gradient of zinc, respectively

$ZnSO_4$: zinc sulfate; *Zn-Met*: zinc-methionine

Determination of Calcium Ion in Cell and Culture Medium

The culture mediums were collected for Ca^{2+} concentration determination using a calcium ion assay kit (Beyotime, S1063S) and a microplate reader. Moreover, the determination of calcium ions in cells was according to the methods described by Zhang et al. [23] In brief, the cells were washed three times by sterile PBS and digested with 0.25% trypsin. DMEM supplemented with 10% FBS was then added to terminate digestion, followed by centrifugation (1500 rpm, 5 min). The cells in sediments were washed twice with PBS and centrifuged again to obtain a cell pellet. Subsequently, cells were incubated with Fluo-4 AM (final concentration of 1 μM) for 30 min at 37 °C, then washed three times with PBS and incubated for an additional 15 min in the absence of Fluo-4 AM (Shanghai Beyotime Bio-Tech Co., Ltd.) to complete the de-esterification process of the dye. Finally, The fluorescent intensity was obtained via flow cytometry.

Analysis of mRNA Expression (Real-time Quantitative PCR)

The epithelial cells were washed three times by PBS. Then, total RNA was isolated from epithelial cells using a Trizol reagent (Invitrogen) in accordance with the manufacturer's instruction. The concentration of each isolated RNA sample was determined using a NanoDrop Spectrophotometer (ND-2000; Gene Company Ltd), and the integrity of the RNA was checked using denatured RNA electrophoresis. A total of 1 μg of RNA was used to obtain complementary DNA by reverse transcription using the HiScript II Q RT SuperMix Reverse Transcriptase (Vazyme). Real-time quantitative PCR reactions were performed on an ABI 7500 real-time quantitative PCR system with a 10-μl reaction volume containing 5 μl of 2× ChamQ™ Universal SYBR-Green qPCR Master Mix (Vazyme), 0.2 μl of 10 μM each of forward and reverse primers, 1 μl diluted complementary DNA template (10-fold), and 3.6 μl double-distilled

H_2O. The primer sequences for SLC39A5 (ZIP5), SLC30A1 (ZnT1), SLC30A5 (ZnT5), metallothionein (MT), metal-response element-binding transcription factor-1 (MTF-1), divalent Metal Transport 1 (DMT1), osteopontin (OPN), ovocleidin-17 (OC-17), ovocleidin-116 (OC-116), ovalbumin (OVA), ovotransferrin (OVOT), Clusterin (CLU), CA, Ca-binding protein-d_{28k} (CaBP-D_{28k}), Ca^{2+}-ATPase, and β-actin are given in Table 2. The protocol of PCR was as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. All reactions were performed in duplicates and experiments were conducted with six replicates per treatment. The $2^{-\Delta\Delta CT}$ was used to calculate the mRNA level of each target gene [24]. The internal reference genes (β-actin) was used to normalize the expression level of the targeted gene.

Statistical Analyses

The results were presented as means with standard errors and analyzed statistically by one-way ANOVA using SPSS 20.0. Differences among all treatments were separated by the Tukey test for multiple comparisons. Values of $P < 0.05$ were considered significant. All the graphs were made using GraphPad Prism 5.01.

Results

Cultivation of Endometrial Epithelial Cells

The uterus scissored is shown in Fig. 1(a). The endometrium is grayish red with long and complex folds. It was observed that the EECs showed adherent growth, with 70–75% adherent growth after 48 h of inoculation. And morphology characteristics of the epithelial cell were polygonal or elliptic with a clear border, arranged closely with full cytoplasm, round nucleus, and then grown as spiral in shape (Fig. 1(b)).

Growth Curve of Epithelial Cells

The EECs were isolated and cultured and then grew slowly within the first 24 h after cell culture. During 2–5 days after cell culture in vitro, EECs were in the logarithmic phase and then reached the plateau phase on the 7th day (Fig. 2).

Effects of Zinc on the Proliferation of Epithelial Cells In Vitro

It was shown that the EECs in vitro were treated with different concentrations and forms of zinc for 24-h or 48-h treatment time (Fig. 3(a), (b)). There were markedly promoted

Table 2 Primers used for real-time quantitative fluorescence PCR analysis

Genes	Forward primer (5′→3′)	Reverse primer (5′→3′)	Size (bp)	Accession no.
ZIP5	CCAAGATGAAACGCACGCAA	GCTGCGACCAAGTCCTGTTA	281	XM_025145573.1
ZnT1	ACCTCCAGACAACCTTTGGTG	AGGAAGAAGGCACTCGCAGA	126	XM_421021.5
ZnT5	ATGGAGGAAAAGTACAGCAGCC	TCAGAAACTTGGCGAAGCAC	118	NM_001031419.2
MT	AACCGACCCGAACTGAAC	CGAAGCATGGCAGCTATTTAC	248	NM_205275.1
MTF-1	CCTGGTTCAACTCCTATGC	TCAAACGGCTTCTCCTTA	278	NM_001031495.1
DMT1	AGCCGTTCACCACTTATTTCG	GGTCCAAATAGGCGATGCTC	129	XM_025145317.1
OPN	GAGCGTAGAGAACGACAGCC	CGCTCTCTAGCGTCTGGTTG	143	NM_204535.4
OC-17	GCAATGCCTTCGTCTGCAAAG	CTGCTGTGGGTCCGTTTATTG	132	KF835610.1
OC-116	GCTTTTGATGAGACTGGACGAG	AGCGGGTAGCGACAACATC	173	AF148716.3
OVA	GCTATGGGCATTACTGACG	TGCTGACCCTACCACCTCT	141	NM_205152.2
OVOT	ATTGCTGCTGAGATTTATG	TTCTATGCCTTCCCACT	204	NM_205304.1
CLU	GCTTCCACCGCCTCTTC	CACTTCTCGCACTCATCCC	208	NM_204900.1
CA	CCTCCGACAAGTCAGTGCTGCAA	TTCAVGCCATCCACAGTGTGCTC	213	Z14957.1
CaBP-D_{28k}	GGTGCCAGCAGCTAAAGTCA	TGGCCAGTTCAGTAAGCTCC	228	EU404189.1
Ca^{2+}-ATPase	ATCGAGGCGCTGAAGGAATA CGAG	CAGCGTGGTGGATTTGATGG AGA	174	NM_205519.1
β-actin	CGCATAAAACAAGACGAGA	GACACCTTCACCATTCCA	91	NM_205518.1

ZIP5: Zrt- and Irt-like protein 5, *SLC39A5*; ZnT1: zinc transporter 1, *SLC30A1*; ZnT5: zinc transporter 5, *SLC30A5*; MT: metallothionein; *MTF-1*: metal-response element-binding transcription factor-1; *DMT1*: Divalent metal transport 1; *OPN*: osteopontin; *OC-17*: ovocleidin-17; *OC-116*: ovocleidin-116; *OVA*: ovalbumin; *OVOT*: ovotransferrin; *CLU*: Clusterin; *CA*: carbonic anhydrase; *CaBP-D_{28k}*: Ca-binding protein-d_{28k}

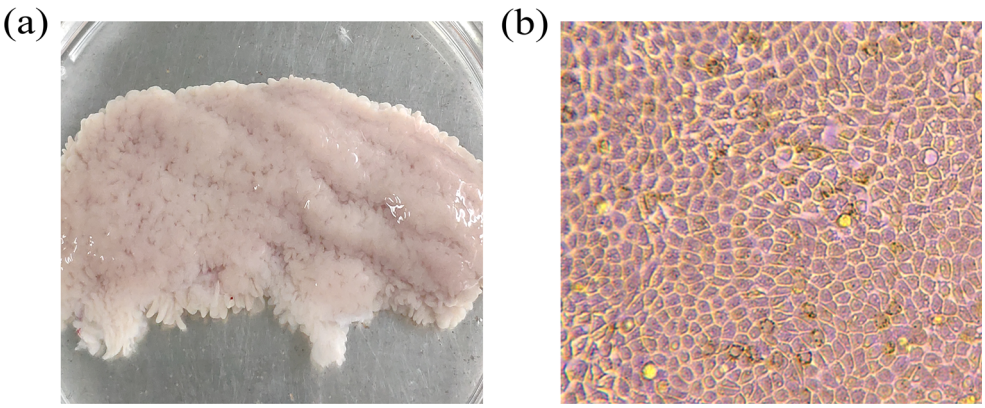

Fig. 1 (Color online) (a) The uterus was cut open with a scissor in separation and cultivation of endometrial epithelial cells; (b) Morphology characteristics of epithelial cell of laying hen after 48 h cultivation using the inverted microscope vision fields (× 4)

proliferation of the EECs treated by 50 μM $ZnSO_4$ or Zn-Met (24 h or 48 h) ($P < 0.05$). Moreover, there was stimulated the proliferation of the epithelial cells at 24-h zinc -treated time.

Determination of Intracellular Carbonic Anhydrase and Calcium Ion in Cell and Culture Medium

The intracellular CA activities and calcium ion in culture medium in EECs in vitro were not affected ($P > 0.05$) by any form of zinc treatment (Fig. 4(a), (b)). The intracellular Ca^{2+} concentration determined by Fluo-4 AM is shown in Fig. 4(c), (d). The relative fluorescence intensity of Ca^{2+} in the Zn-Met group was significantly higher than that in the control group ($P < 0.05$), but there was no significant difference between the Zn-Met group and the $ZnSO_4$ group ($P > 0.05$).

Effects of Zinc on the mRNA Expressions of Zinc Transporters and Genes Involved in Calcium Deposition in Endometrial Epithelium Cells of Laying Hen In Vitro

As for zinc transporters, it was only observed that mRNA levels of MT in EECs in vitro showed a significant difference ($P < 0.05$) between the Zn-Met group and the control (Fig. 5).

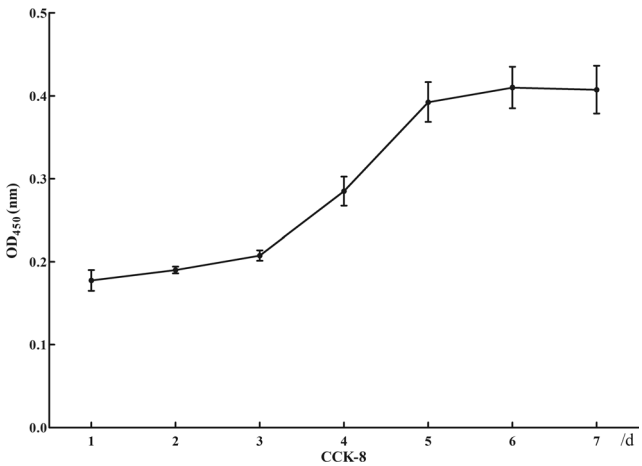

Fig. 2 Growth curves of endometrial epithelial cells of laying hen in vitro

However, no significant ($P > 0.05$) effects among the three groups were observed in mRNA levels of OPN, OC-17, OC-116, OVA, OVOT, CLU, CA, Ca^{2+}-ATPase, and $CaBP\text{-}D_{28k}$ in EECs in vitro (Fig. 6).

Discussion

In mammals, the womb is where the embryo is implanted and develops, and successful culture of primary EECs in vitro has been widely used to study the relationship between embryo and endometrium and the importance of endometrium to embryonic development and survival [25, 26]. But, for birds, the uterus is vesicular shaped and is an important part for eggshell mineralization, which secretes the calcium, pigments, and matrix proteins during eggshell formation [4]. It was observed that when the eggshell was being formed in the uterus of laying hens, the endometrium became thinner, the bulges of the wrinkled wall became shorter, and the uterus was hyperemic while the wall of the uterus without eggshell formation was thick, with long and dense wrinkled bulges, and the vesicle shape of the uterus became smaller. Therefore, the endometrium plays an important role not only in the study of mammalian reproductive physiology but also in the mechanism of eggshell formation in laying hens.

At present, there are few studies on avian EECs in vitro. Kai and Mineo [27] and Kai [28] studied the culture of EECs of Japanese quail in vitro and related experiments of pigment secretion on the cultured cells. Sun et al. [11] studied the effects of steroid hormones on the culture and proliferation of EECs in laying hens in vitro. In order to further study the mechanism of Zn-Met regulating eggshell quality in vitro, the current experiment refers to the culture method of EECs reported in the above study, and was to establish a model of EECs of laying hens, which would be a certain foundation for subsequent experiments.

In the present study, the EECs of laying hens were isolated and cultured by the scraping method and the digestion method of type I collagenase which was consistent with that of EECs

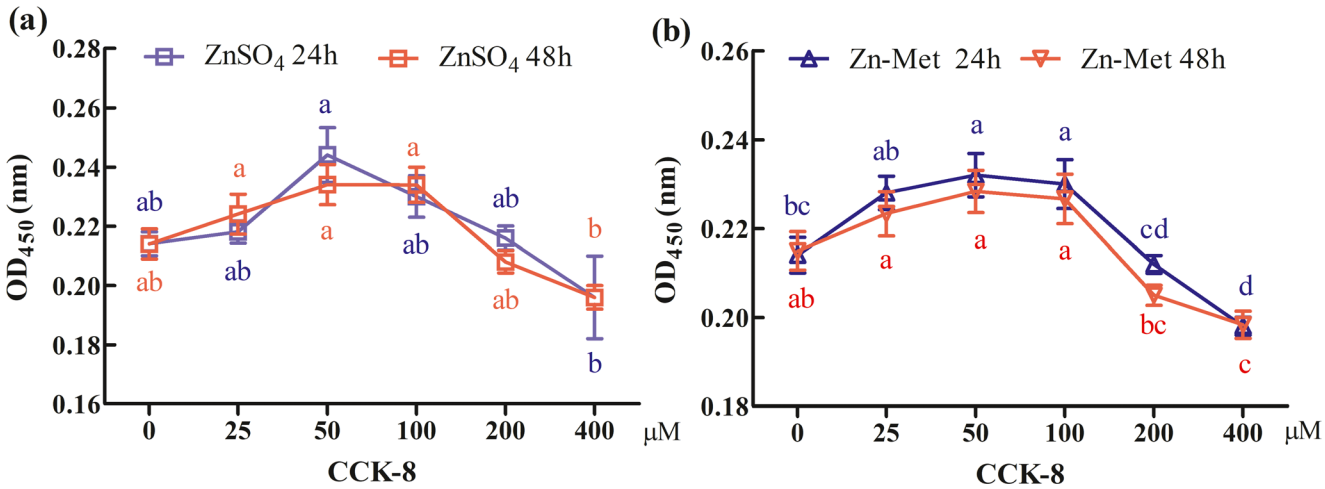

Fig. 3 Effects of zinc on the proliferation of epithelial cells of laying hen in vitro. On the third day of cultivation, different forms and levels of zinc were added to the medium, and effects of zinc on the proliferation of epithelial cells were determined by using the CCK8 assays at 24-h and 48-h zinc treatment time, respectively

cultured in layers by Sun et al. [11] Compared with the study of Sun et al. [11], the process of establishing our cell culture model has a centrifugal process to remove impurities before digestion, and we use a three-layer gauze instead of a cell screen for filtration after terminating digestion. The process of cell culture was more detailed and specific. It was found that after 48 h of cultivation, the EECs were adherent up to 70–75%, and the cell morphology is polygonal or elliptic with clear boundary, close arrangement, and a vortex-shaped growth, which was the same as the EEC morphology isolated and identified by Sun et al. [11] In addition, it is important that hens used for sampling should be during the peak of laying. When hens were young, the culture of EECs was not ideal, and the proliferation and differentiation ability of EECs was not very good. It was shown that the adherent cells were less and the cell apoptosis was fast. During cell separation, it should be noted that excess red blood cells could affect adherence activity in cell culture. In addition, the glucose content of the medium used in cell culture should not be too high. In our experiment, it was observed that when a high-glucose medium was used, the cell survival time was not long. In the current study, it was found that the EECs obtained by type I collagenase digestion grew well and could survive for 8 days, in which the growth reached the logarithmic stage on day 2 and reached the growth plateau on days 6 to 7. This result was also consistent with that of EECs cultured in layers by Sun et al. [11]

It has been demonstrated that metal chelated with amino acid or protein has a better bioavailability to poultry [29, 30], but the study on amino acid–chelated zinc in EECs in laying hens in vitro has not been seen. According to the experiments conducted by Sauer et al. [31] and Huang et al. [32] to study the absorption characteristics of amino acid–chelated zinc in cell lines, the present experiment was designed with five additive concentration gradients of zinc. In this experiment, it was found that the EECs on the third day of cultivation showed stronger cell activity and better growth after the exogenous addition of 50 μM zinc ($ZnSO_4$ or Zn-Met) for 24 h. This result was consistent with the biological function of zinc, which is involved in regulating the expression and activation of various biomolecules, such as transcription factors, enzymes, channels, growth factors, and their associated receptors. However, when zinc is deficient or excessive, it will affect growth, morphology, immune response, and neuroendocrine activities [33].

In the present experiment, it was found that intracellular CA activity and Ca^{2+} concentration in culture medium were not affected by the treatment with 50 μM Zn concentration for 24 h, while intracellular Ca^{2+} concentration in the Zn-Met group was the highest and significantly higher than the control group, which was consistent with the previous experimental results in vivo [22] that CA activity in ESG is unaffected and Ca^{2+} concentration in ESG is highest in the Zn-Met group. This suggests that Zn-Met does increase the concentration of Ca^{2+} in ESG, so that more calcium ions can be used for transport into the uterine cavity to form eggshells.

To explore the characteristics of Zn-Met absorbed and transported in EECs, the zinc transport–related gene expression levels were measured. It was found that Zip5, ZnT1, ZnT5 and MTF-1, and DMT1 mRNA expression levels were not affected by zinc processing, only MT mRNA expression was highest in the Zn-Met group, which showed that Zn-Met can regulate the expression of MT in layers, and the mechanism of zinc absorbed in the intestine and transported in the uterus is mainly associated with MT mRNA expression. Exogenous zinc added at 50 μM may be enough for EEC of layers, so it was no significant difference between the Zn-Met

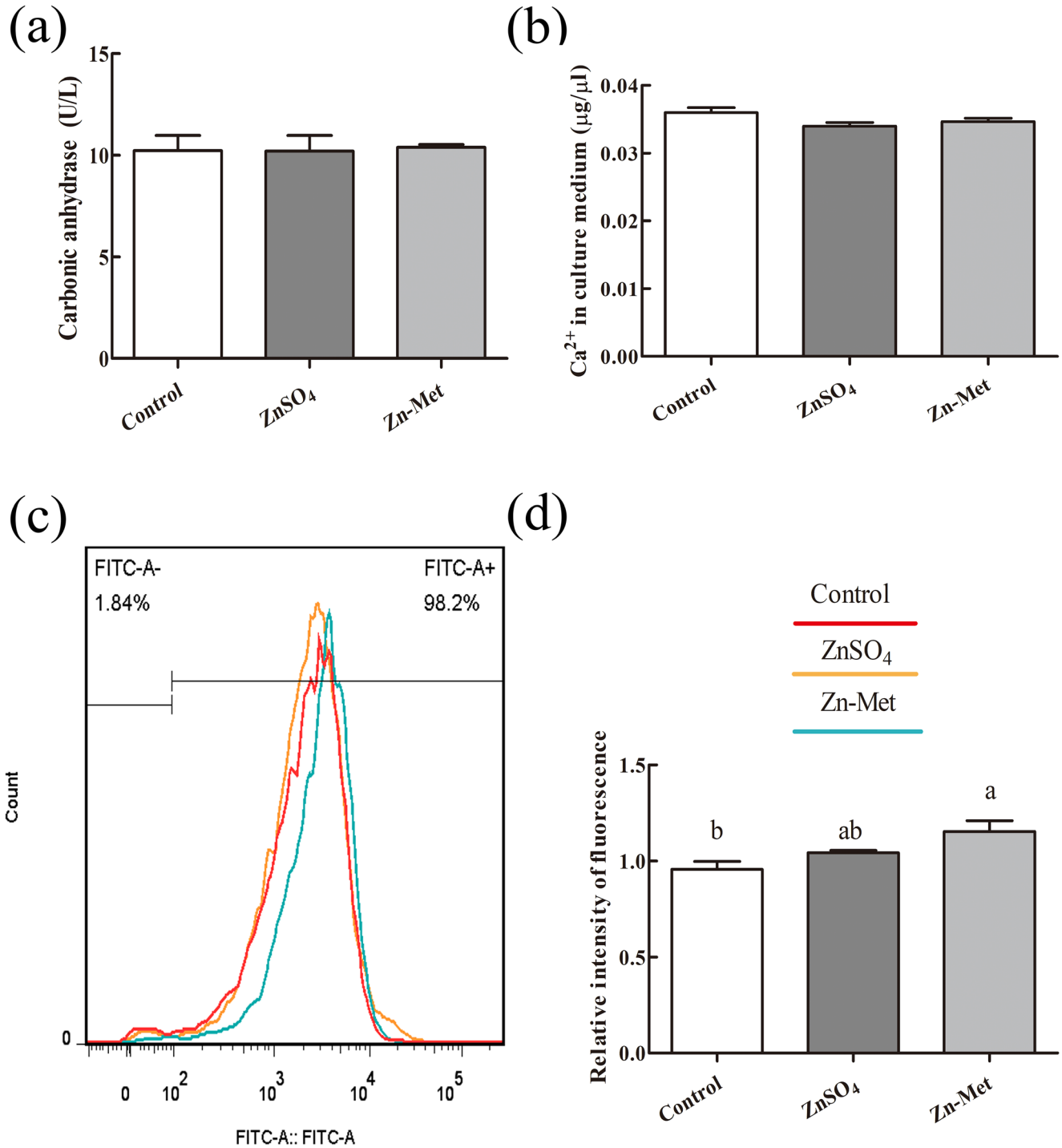

Fig. 4 (Color online) Effects of zinc on the intracellular CA activity and calcium ion in cell and medium in endometrial epithelium cells of laying hen in vitro. (a): cellular CA activity determined using ELISA kits; (b): Ca^{2+} concentration in culture medium determined using calcium ion assay kit; (c), (d): the endometrial epithelium cells were treated with different forms of zinc for 24 h, the cytosolic Ca^{2+} was stained with Fluo-4 AM and determined by flow cytometry. Values are means (six samples per treatment) with standard errors represented by vertical bars. [a,b,c]Mean values with different letters were significantly different among the three treatments ($P < 0{\cdot}05$). Control, no zinc treated; $ZnSO_4$, zinc sulfate at 50 μM; Zn-Met, Zn-Met at 50 μM. CA: carbonic anhydrase

group and the $ZnSO_4$ group in other zinc transporter expression. It also suggested that the absorption characteristics of amino acid–chelated zinc in the body was mainly in the form of zinc ions by saturable zinc transporter [34]. As the transporters approach saturation, other transport ways start to be mobilized.

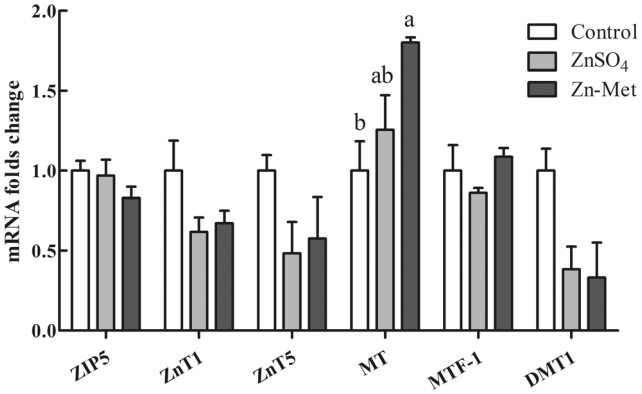

Fig. 5 Effects of zinc on the mRNA expressions of zinc transporters in endometrial epithelium cells of laying hen in vitro. Values are means (six samples per treatment) with standard errors represented by vertical bars. [a,b,c]Mean values with different letters were significantly different among the three treatments ($P < 0{\cdot}05$). Control, no zinc treated; $ZnSO_4$, zinc sulfate at 50 μM; Zn-Met, Zn-Met at 50 μM. ZIP5: Zrt- and Irt-like protein 5, SLC39A5; ZnT1: zinc transporter 1, SLC30A1; ZnT5: zinc transporter 5, SLC30A5; MT: metallothionein; MTF-1: metal-response element-binding transcription factor-1; DMT1: Divalent metal transport 1

In addition, our study found that zinc treatment had no significant effect on the mRNA expression levels of OPN, CLU, OVA, OVOT, OC-17, OC-116, CA, Ca^{2+}-ATPase, and CaBP-D_{28k} in EECs. The results were inconsistent with the previous results in vivo that the CA and CaBP-D_{28k} mRNA expression in ESG of the Zn-Met group were significantly higher than that of the control group. Considering about 20 h taken in the shell formation, this difference might be because a single cell had limited function, and was not enough to complete the generation and regulation of necessary substances in the entire process of shell formation. Therefore, the expression of eggshell formation–related genes on the EECs of laying hens needs further study.

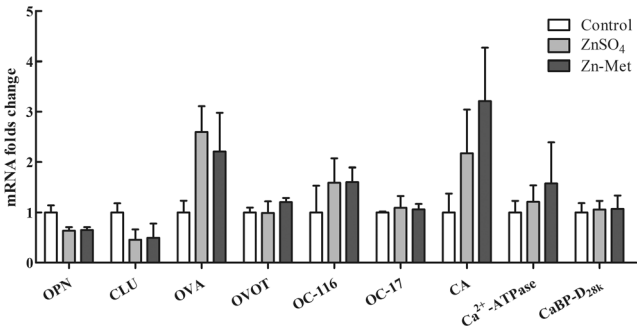

Fig. 6 Effects of zinc on the mRNA expressions of genes involved in calcium deposition in endometrial epithelium cells of laying hen in vitro. Values are means (six samples per treatment) with standard errors represented by vertical bars. Control, no zinc treated; $ZnSO_4$, zinc sulfate at 50 μM; Zn-Met, Zn-Met at 50 μM. OPN: osteopontin; OC-17: ovocleidin-17; OC-116: ovocleidin-116; OVA: ovalbumin; OVOT: ovotransferrin; CLU: Clusterin; CA: carbonic anhydrase; CaBP-D_{28k}: Ca-binding protein-d_{28k}

Conclusions

In conclusion, the EECs of laying hens isolated by scraping and digested collagenase I were with better adherent growth. The model establishment of the EECs plays an important role in the study of uterine function in poultry. Moreover, Zn-Met can increase intracellular Ca^{2+} concentration and upregulate expressions of MT mRNA in the EECs of laying hens.

Author Contributions Lanlan Li conceived and designed the experiments. Lanlan Li, Wenting Zhou, and Sasa Miao conducted them and took part in the sample collection. Lanlan Li conducted the laboratory experiments, analyzed the data, and wrote the manuscript. Xiaoting Zou and Xinyang Dong revised the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there are no conflicts of interest.

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