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Effects of Calcitonin on Porcine Intestinal Epithelial Cells Proliferation, Phosphorus Absorption, and NaPi-IIb Expression

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ABSTRACT

To investigate the effects of calcitonin (CT) on cell proliferation, NaPi-IIb expression, and the absorption of phosphorus in porcine intestinal epithelial cells (IPEC-1). The experiment consisted of 1 control treatment and 5 levels of CT treatments (1×10^{-12} to 1×10^{-8} mol/L), and each treatment has 6 replications. Cell proliferation was determined by the MTT method. Cell morphology and phosphorus concentration in the cell supernatant were measured after 24 h of CT treatment. The NaPi-IIb protein expression was determined by Western Blot, and the *NaPi-IIb* mRNA expression was determined by RT-PCR. The results showed that, compared with the control group, different levels of CT had no effect on cell proliferation, but it inhibited ($P < 0.05$) the absorption of phosphorus at CT concentration of 1×10^{-11}, 1×10^{-10} mol/L and 1×10^{-9} mol/L. There was no effect of CT on *NaPi-IIb* mRNA and NaPi-IIb protein expression. The study suggested that CT can inhibit the absorption of phosphorus in porcine intestinal epithelial cells without affects NaPi-IIb expression.

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Authors' Contribution
XT and WS designed and performed the experiment. XT wrote the experiment. RF provided the technical and financial support and revised the paper.

Key words
Phosphorus absorption, Calcitonin, NaPi-IIb, Porcine intestinal epithelial cell.

INTRODUCTION

Inorganic phosphate (Pi) plays a major role in growth, development, bone formation and cellular metabolism. Pi absorption from small intestine and reabsorption from rental play key roles in phosphate homeostasis (Xiang *et al*., 2012). Phosphorus is mainly absorbed by simple diffusion and active absorption. Many researchers have demonstrated that the active absorption of Pi is mediated by sodium-dependent transport family protein NaPi-IIb (Segawa *et al*., 2004; Murer *et al*., 2004; Dërmaku-Sopjani *et al*., 2011; Xiang *et al*., 2012; Forster *et al*., 2012), which accounts for 70% to 90% of the body phosphorus active absorption (Sabbagh *et al*., 2009; Wong *et al*., 2012). NaPi-IIb expression *in vivo* is regulated by many physiological factors, such as estrogen (Xiang *et al*., 2012; Fang *et al*., 2016), epidermal growth factor (EGF) (Xing *et al*., 2017), glucocorticoid (Buller *et al*., 2011), vitamin D_3 and its intermediate 1,25(OH)2D_3 (Cohen *et al*., 2009), insulin-like growth factors (IGF) (Zhang *et al*., 2010), and protein kinase including Serum and Glucocorticoid inducible Kinase (SGK) (Rosario *et al*., 2013). Thus, investigating the regulatory factors of NaPi-IIb deeply is critically important for improving intestinal phosphorus utilization, decreasing manure phosphorus excretion and reducing environmental pollution. Calcitonin (CT) is an important regulator hormone of calcium and phosphorus in the body, and can reduce the absorption of phosphorus (Matsui *et al*., 1983). Early studies about CT were focused on the influence of osteoclasts and calcium metabolism (Naot and Cornish, 2008; Broulik, 2010). Studies on Pi uptake and NaPi-IIb expression mediated by CT in the small intestine are still lacking. Therefore, the objective of this study was to investigate the effects of CT on IPEC-1 growth, NaPi-IIb expression, and absorption of phosphorus, to further elucidate the mechanism of Pi absorption *in vitro*.

MATERIALS AND METHODS

Cell culture

Porcine intestinal epithelial cells (IPEC-1) were kindly provided by Institute of Subtropical Agriculture, Chinese Academy of Science (Changsha, China). IPEC-1 cells were cultured in DMEM/F12 medium (Invitrogen, Grand Island, NY, USA) containing 10% FBS (HyClone, South Logan, Utah, USA), 1% antibiotics (Penicillin-Streptomycin) (Santa cruz, CA, USA), and grown in a humidified incubator at 37°C with 5% CO_2 and 95% air.

Determination of cell proliferation

To determine the effects of CT on IPEC-1 proliferation, cells were seeded in 96-well cell culture

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plates with approximately 1×10^4 cells per well, and cultured in DMEM-F12 containing 0 mol/L, 1×10^{-12} mol/L, 1×10^{-11} mol/L, 1×10^{-10} mol/L, 1×10^{-9} mol/L or 1×10^{-8} mol/L of CT (GDBIO, Qingdao, China). After a 24 h of culture, the cells were observed under inverted microscope (XDS-1A, Shanghai Precision Instrument Co., Ltd., Shanghai, China). The cell proliferation was determined by methyl-thiazolyl-tetrazalium (MTT) method (Chang *et al*., 2017). At each time point (24, 48, 72, 96h) 20 μL of MTT (Amresco, Solon, OH, USA) was added to each well and further cultured in 37°C, 5% CO_2 atmosphere for 4 h. Removed the supernatant, rinsed the plates twice with PBS buffer (Solarbio, Beijing, China), added 150 μL dimethylsulfoxide (DMSO) (Amresco, Solon, OH, USA) in each well, shaken the plates for 15 min at room temperature to dissolve the blue-violet crystals (Formazan), then measured the optical density (OD) at a wavelength of 490 nm by enzyme-linked immune detector (Bio-Rad, Hercules, CA, USA).

Phosphorus absorption analysis

To determine the effects of CT on phosphorus absorption, cells were seeded in 6-well cell culture plates with approximately 1×10^5 cells per well, and cultured in DMEM-F12 containing 0 mol/L, 1×10^{-12} mol/L, 1×10^{-11} mol/L, 1×10^{-10} mol/L, 1×10^{-9} mol/L or 1×10^{-8} mol/L of CT. After a 24 h of culture, the supernatant was collected in 0.5 mL centrifuge tube for phosphate analysis according to the guidelines of biochemical reagents (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbed phosphorus of cells equals to phosphorus in the medium minus the phosphorus in supernatant.

Real-time PCR (RT-PCR) analysis of NaPi-IIb expression

To determine the effects of CT on *NaPi-IIb* expression, cells were seeded in 6-well cell culture plates with approximately 1×10^5 cells per well, and cultured in DMEM-F12 containing 0 mol/L, 1×10^{-12} mol/L, 1×10^{-11} mol/L, 1×10^{-10} mol/L, 1×10^{-9} mol/L or 1×10^{-8} mol/L of CT. After a 24 h of incubation, total cell RNA was extracted and purified according to the guidelines of biochemical kit (Tiandz Inc., CAT #: 71201-50, Beijing, China). Total RNA was quantified at an OD of 260 nm, and its purity was assessed by determining the OD260/OD280 ratio. Meanwhile, RNA integrity in each sample was determined using 1 % denatured agarose gel electrophoresis. Total RNA was reverse transcribed using a PrimeScript® RT reagent kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instruction. cDNA was synthesized and stored at -20°C.

The primers used to detect *NaPi-IIb* were designed from the reported porcine *NaPi-IIb* sequence (Xiang *et al*., 2012), and the primers used to detect *β-actin* were designed from the reported porcine (Susscrofa) *β-actin* cDNA sequence (U07786) with software Primer 5.0. All primers were synthesized by Shanghai Biological Engineering Company (Shanghai, China). The forward primer of *NaPi-IIb*: 5'-CAGCCCGAAACAAGAGTATGAT-3'; the reverse primer of *NaPi-IIb*: 5'-TACAAACCATCCGTCCAACAG-3'; the forward primer of *β-actin*: 5'-CTTCCTGGGCATGGAGTCCT-3'; The reverse primer of reverse: 5'-CGTGTTGGCGTAGAGGTCCTT-3. The RT-PCR was performed using the SYBR® Premix Ex Taq™ (Takara, Dalian, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA). The total volume of PCR reaction system was 25 μL. Briefly, 12.5 μL SYBR® Premix Ex Taq™, 4 μLcDNA, 1 μL (10 mmol/L) forward/ reverse primers and 8.5 μL dH_2O. All PCRs were performed in triplicate on a 96-well RT-PCR plate under the following conditions: 95°C for 30s followed by 39 cycles of 95°C for 5s, 58°C for 30s and 72°C for 60s.

Western blotting analysis of NaPi-IIb protein

To determine the effects of CT on NaPi-IIb expression, cells were seeded in 6-well cell culture plate with approximately 1×10^5 cells per plate, and cultured in DMEM-F12 containing 0 mol/L, 1×10^{-12} mol/L, 1×10^{-11} mol/L, 1×10^{-10} mol/L, 1×10^{-9} mol/L or 1×10^{-8} mol/L of CT. After a 24 h of incubation, cells were obtained for analysis of protein and Western blotting. Cells in 6-well culture plates were gently washed for 3 times with pre-cooled PBS. Cells were collected and lysed in RIPA Lysis Buffer (containing 1% PMSF, Beyotime, Haimen, China). Cellular debris were removed through centrifugation at 12,000 g at 4°C for 10 min, and the total protein concentration was detected using the Bradford Protein Assay Kit according to the manufacturer's instructions (Beyotime, Haimen, China). The extracted protein sample was denatuned at 100°C for 5 min in a loading buffer containing 5% β- mercaptoethanol and stored at -20°C.

Protein samples were separated by 10% SDS-PAGE, and then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes with transferred proteins were blocked for 1 h at room temperature in blocking buffer containing 5 % Skim-milk in TBST buffer (1× Tris-buffered saline including 0.1 % Tween 20). Membranes were incubated overnight at 4°C with following primary antibodies: rabbit anti-human SLC34A2 antibody (1:200, Santa Cruz Biotechnology, CA, USA), or α-Tubulin (1:1,000, Santa Cruz Biotechnology, CA, USA). After washed 3 times with TBST, the membranes were incubated for 1 h at room temperature with goat anti- rabbit IgG secondary antibody (Anti-Rabbit IgG (H+L) Antibody, 1:1,000, Proteintech

Rosemont, IL, USA). Membranes were washed 3 times with 1 × TBST and colored with a chemiluminescence reagent (ECL-plus, Beyotime, Haimen, China), and the chemifluorescence was detected using the Image Quant LAS 4000 mini system (GE Healthcare Bio-sciences AB, Inc., Sweden), and quantified using a gel-imaging system with Image Quant TL software (GE Healthcare life Science, Inc., USA). Abundances of all proteins of interest were normalized to those for α-Tubulin.

Statistical analysis

Data were analyzed by One-way ANOVA analysis using the SAS software version 9.2 (SAS Institute Inc., NC, USA). All treatments were subjected to linear and quadratic regression analysis of hosphorus absorption to CT concentration. All values were expressed as mean±standard deviation (SD). Differences among treatment mean were determined using Duncan's multiple comparison test, $P < 0.05$ was considered significant.

RESULTS

Effect of CT on IPEC-1 cells proliferation

The morphology of IPEC-1 cells in 0 mol/L, 1×10^{-12} mol/L, 1×10^{-11} mol/L, 1×10^{-10} mol/L, 1×10^{-9} mol/L and 1×10^{-8} mol/L CT group are shown in Figure 1. After a 24 h of culture, all cells nearly had same cell morphology. It suggested that different concentration CT had no effects on the proliferation of cells. The growth curves of IPEC-1 cells are shown in Figure 2. There are no differences of OD value among 0 mol/L, 1×10^{-12} mol/L, 1×10^{-11} mol/L, 1×10^{-10} mol/L, 1×10^{-9} mol/L and 1×10^{-8} mol/L CT groups in 24, 48, 72 or 96 h. It indicted that CT had no effect on proliferation of IPEC-1 cells.

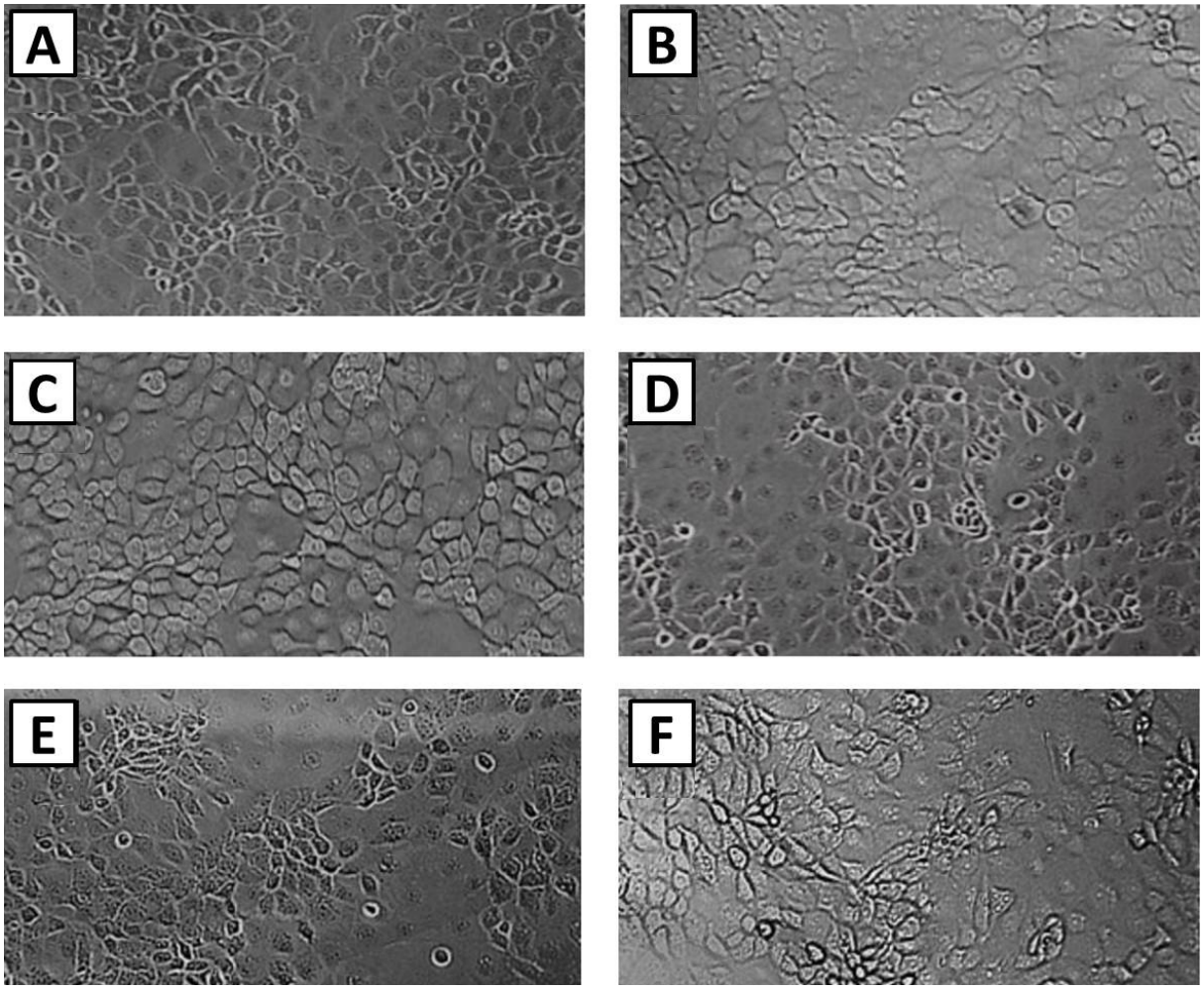

Fig. 1. Cell morphology of IPEC-1 after 24 h of culture with different CT levels (×100). A, control group (no CT); B, 1×10^{-12} mol/L CT treated; C, 1×10^{-11} mol/L CT treated; D, 1×10^{-10} mol/L CT treated; E, 1×10^{-9} mol/L CT treated; F, 1×10^{-8} mol/L CT treated.

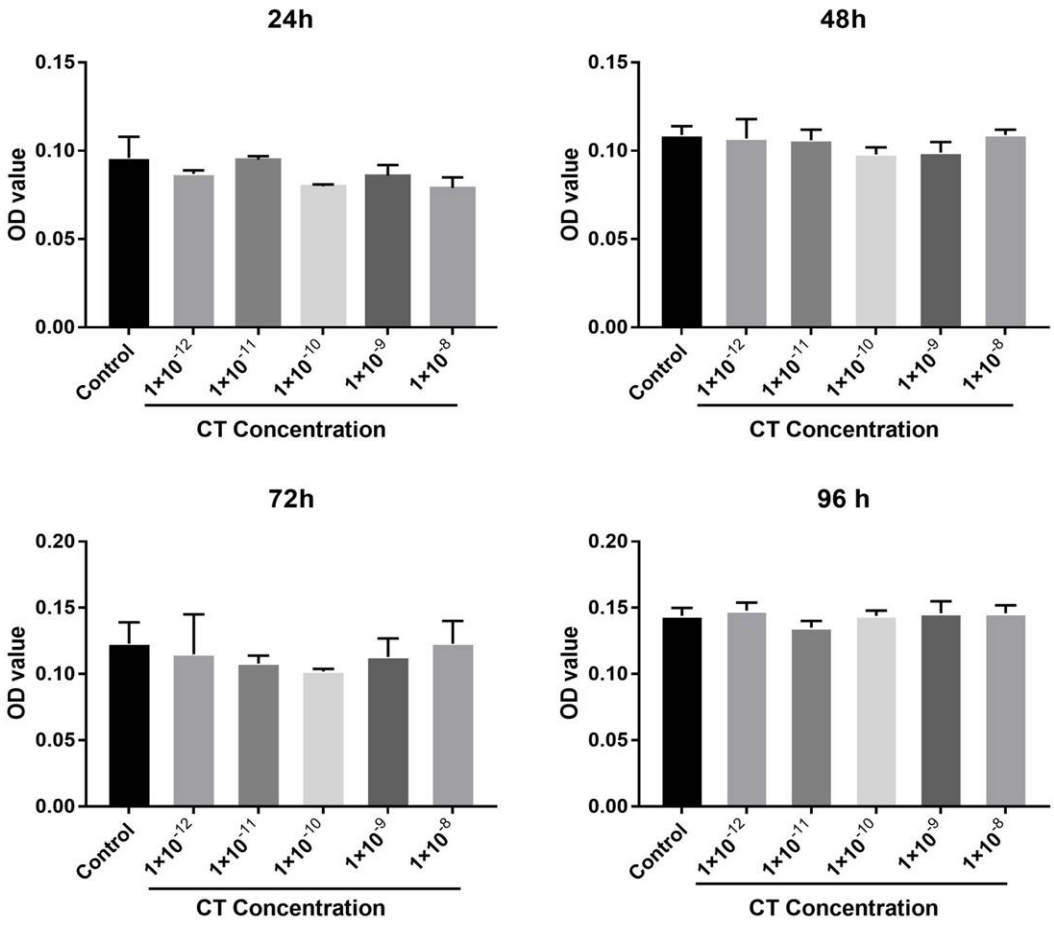

Fig. 2. Effect of CT on IPEC-1 cell growth. Data are means±SD, n=6. Bars without letter means no significant difference ($P > 0.05$).

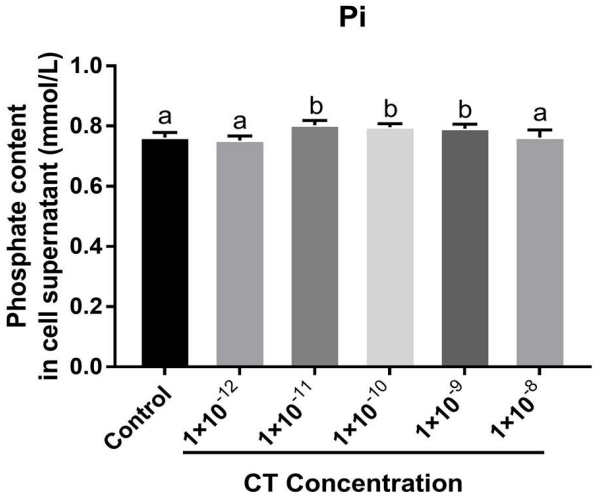

Fig. 3. Effect of CT on phosphorus absorption. Data are means ± SD, n=6. a, b means sharing different letters differ ($P < 0.05$).

Effects of CT on phosphorus absorption

Effect of CT on phosphorus absorption was shown in Figure 3. The results showed that groups of 1×10^{-9} mol/L, 1×10^{-10} mol/L and 1×10^{-11}mol/L had a lower phosphorus absorption compared with control group ($P < 0.05$). 1×10^{-8} mol/L and 1×10^{-12} mol/L groups have no difference compared with control group ($P > 0.05$). What else, there was a tendency of linear relationship between phosphorus absorption to CT concentration (P=0.073), and there was a significant quadratic relationship between phosphorus absorption to CT concentration ($P< 0.05$). The results indicated that 1×10^{-11}mol/L to 1×10^{-9} mol/L CT had an inhibition effect on phosphorus absorption.

Effect of CT on NaPi-IIb mRNA expression

After 24 h of culture, the cell total RNA was extracted, and the concentration total RNA was detected, all samples had an OD_{260}/OD_{280} value between 1.9 -2.1, then 1% agarose gel electrophoresis showed that all samples had a

brightness 28s and 18s band (Fig. 4). The *NaPi-IIb* gene expression in different levels of CT treated was showed in Figure 4. Compared with control group, adding 1×10^{-12}, 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} mol/L CT decreased the mRNA level of *NaPi-IIb* for 12.84%, 44.28%, 22.98%, 5.07%, 32.54%, respectively, but the difference was not significant.

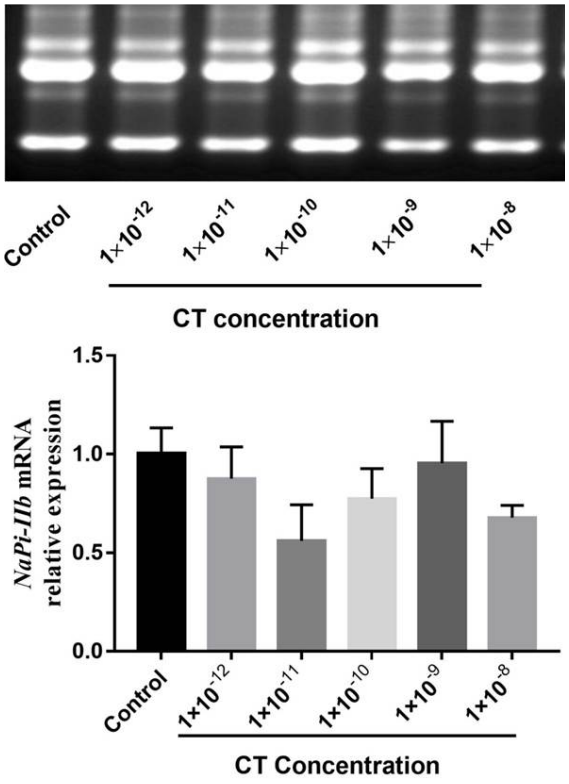

Fig. 4. Effects of CT treatment on expression of *NaPi-IIb* gene. Data are means±SD, n=6. Bars without letter means no significant difference ($P > 0.05$).

Effect of CT on NaPi-IIb protein expression

The NaPi-IIb protein expression was showed in Figure 5. Compared with control group, adding 1×10^{-12}, 1×10^{-11}, 1×10^{-10}, 1×10^{-9}, 1×10^{-8} mol/L CT decreased the NaPi-IIb protein expression for 1.44%, 1.38%, 0.53%, 0.81%, 2.95%, respectively, but the difference was not significant. It suggested that CT had no effects on NaPi-IIb protein expression.

DISCUSSION

Effect of CT on intestinal epithelial cells proliferation

Cell morphology and growth trend could reflect the effects of drugs on cell differentiation and cell vitality. In present study, we treated the cells with different levels of CT for 24 h, observed under an inverted microscope, and found that cells were in a good condition all with a clear edge. MTT colorimetric method was usually used to measure cell proliferation, to detect cell survival and growth, and to investigate whether drugs treated have impacts on cell proliferation (Lu *et al*., 2012). MTT was a yellow dyestuff which can react with mitochondria succinate dehydrogenase of viable cells to generate violet crystal formazan, and deposited in cells, while dead cells have no this feature. Formazan was water-insoluble, but can dissolve in dimethyl sulfoxide (DMSO). The optical density value measured at the wavelength of 490 nm (or 570 nm) can serve as an estimation of the number of living cells indirectly. In present study, there are no differences of OD value among 0 mol/L, 1×10^{-12} mol/L, 1×10^{-11} mol/L, 1×10^{-10} mol/L, 1×10^{-9} mol/L and 1×10^{-8} mol/L CT groups in 24, 48, 72 or 96 h. The results suggested that there were no significant effects of CT on porcine intestinal epithelia cell morphology and proliferation, and it also can explain the changes of phosphorus content in cell supernatants was not caused by cell viability and quantities in subsequent experiment.

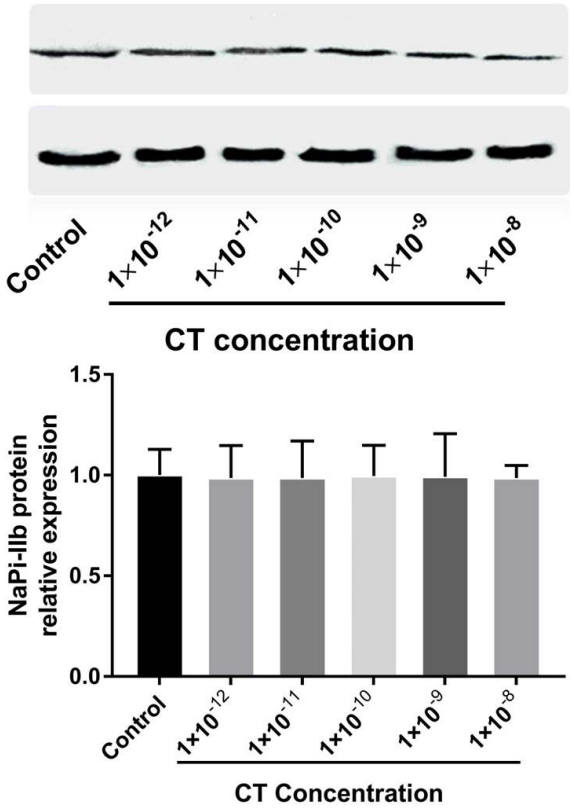

Fig. 5. Effects of CT treatment on expression of NaPi-IIb protein. Data are means±SD, n=3. Bars without letter means no significant difference ($P > 0.05$).

Effects of CT on phosphorus absorption

As an important component of the body, phosphorus is one of main element in bone, and involved in a variety of physiological functions (Fang *et al*., 2012, 2016). CT was a 32 peptide hormone secreted by para-follicular cells (C cells) in thyroid, which involved in calcium and phosphate regulation, osteoclast activity inhibition and blood calcium reduction (Kanis, 2002); inhibiting the absorption of calcium and phosphate in intestine (Han *et al*., 2012). CT was an important hormone for calcium and phosphorus regulation, as well as an effective drug for the treatment of bone hyperplasia. In this study, the absorption of phosphorus can be measured via detecting the content of phosphorus in culture supernatant, which the content of phosphorus in medium was known (0.902 mmol/L). The results showed that 1×10^{-9} mol/L, 1×10^{-10} mol/L and 1×10^{-11} mol/L of CT can inhibit the phosphorus absorption.

Effects of CT on NaPi-IIb expression

As the major sodium-dependent phosphate transporter protein in intestine, *NaPi-IIb* plays an important role in Pi active absorption (Reining *et al*., 2010), and it was responsible for intracellular Pi accumulation and phosphate homeostasis (Wang and Yin, 2009). The expression of *NaPi-IIb* was related to transcription factors, protein translation and intracellular transport, and influenced by many physiological factors, such as age (Xu *et al*., 2002), diets phosphorus levels (Fang *et al*., 2012), intestinal segment (Radanovic *et al*., 2005), estradiol (Xu *et al*., 2003a), glucocorticoid (Arima *et al*., 2002), 1α-OHD_3 (Marks *et al*., 2006), EGF (Xu *et al*., 2003b; Xing *et al*., 2017), B-RAF (Pakladok *et al*., 2014) and neuropeptides (Hu and Fang, 2014) and so on. Early studies about CT were focused on the influence of osteoclasts and calcium metabolism (Naot and Cornish, 2008; Broulik, 2010), only Berndt (1992) suggested that in the absence of PTH and cAMP, CT reduced the sodium dependent phosphate transporter protein on the proximal tubule brush border membrane by increasing the intracellular concentration of calcium. In present study, porcine intestinal epithelial cells was used as cell model to detect the effects of CT on expression of NaPi-IIb protein and *NaPi-IIb* mRNA. The results suggested that there was no effect on expression of NaPi-IIb protein and *NaPi-IIb* mRNA. It suggested that the absorption of phosphorus in cells regulated by CT was not mediated by NaPi-IIb. In body, CT was usually worked with PTH and 1,25-$(OH)_2D_3$ to maintain the balance of blood calcium and phosphorus (Li and Yuan, 2013). PTH reduced the absorption of phosphorus by inhibiting the *NaPi-IIb* expression in intestine epithelium (Tenent and Ouse, 2005). 1,25-$(OH)_2D_3$ can simulate brush border *NaPi-IIb* expression to promote the absorption of phosphorus (Marks *et al*., 2006). Hence, CT may inhibit intestine phosphorus absorption by changing PTH and 1, 25-$(OH)_2D_3$ expression indirectly.

CONCLUSION

The results of this research suggested that the concentration of CT range from 1×10^{-12}mol/L to 1×10^{-8}mol/L has no effects on porcine intestinal epithelial cells growth; and 1×10^{-11}mol/L CT to 1×10^{-9}mol/L CT can inhibit the phosphorus absorption; also there was no effects of the concentration of CT range from 1×10^{-8}mol/L to 1×10^{-12}mol/L on NaPi-IIb expression. The present study suggested that CT can inhibit the absorption of phosphorus in porcine intestinal epithelial cells without affects NaPi-IIb expression.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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