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Acute Effect of Epidermal Growth Factor on Phosphate Diffusion across Intestinal Mucosa of Hens using the Ussing Chamber System

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XT and RF designed and performed the experiments. XT wrote the paper. GP and KX helped in writing the article.

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ABSTRACT

The aim of this study was to investigate the effect of epidermal growth factor (EGF) on phosphate absorption across intestinal mucosa of hens using the Ussing chamber system. First, four Loman laying hens with similar weight (1.25±0.13 kg) were sacrificed to detect the lactate dehydrogenase (LDH) activity of isolated small intestine at time on 0 min, 30 min, 60 min, 90 min and 120 min, to provide a appropriate operation time for Ussing chamber test. Then, a total of 20 Loman laying hens with similar weight (1.25±0.13 kg) were divided into 1 of 5 treatments: i) control group (0 ng/mL EGF), ii) 50 ng/mL EGF group, iii) 100 ng/mL EGF group, iv) 150 ng/mL EGF group, and v) 200 ng/mL EGF group for using chamber test. The results showed that (i) the LDH activity of isolated intestine increased ($P < 0.05$) sharply during 30 min, at 60 min, the LDH activity of isolated intestines reached a high level; (ii) EGF increased ($P < 0.05$) the transepithelial electrical resistance (TEER, $\Omega.cm^2$) significantly; (iii) EGF inhibited ($P < 0.05$) the phosphate diffusion across intestinal mucosa significantly. According to the results, the Ussing chamber system can use to evaluate phosphate diffusion across intestinal mucosa, and EGF may inhibit the absorption of phosphorus.

INTRODUCTION

Phosphate (Pi) is a key component of biological systems involved in a variety of physiologic processes including energy metabolism, cell signaling, nucleotide and phospholipids biosynthesis, and bone mineralization (Fang *et al*., 2012; Giral *et al*., 2012). Pi is absorbed by the epithelium of the small intestine via both a passive diffusion and an active sodium-dependent process (Penido and Alon, 2012; Fang *et al*., 2016). Previous studies have demonstrated that the active absorption of Pi across intestinal epithelium is mainly mediated by type IIb sodium-dependent transport (NaPi-IIb) protein (Xiang *et al*., 2012; Fang *et al*., 2012, 2016).

EGF is a small mitogenic polypeptide comprising 53 amino acid residues, which has established as a trophic factor for the intestinal maturation, epithelial cell homeostasis, and ions transport in the small intestine (Trapani *et al*., 2014; Tang *et al*., 2016, 2018a). Previous studies have demonstrated that EGF inhibited the expression of NaPi-IIb (Xu *et al*., 2001, 2003; Xing *et al*., 2017; Tang *et al*., 2018b), which implied that EGF inhibited the active absorption of Pi. However, there is no direct evidence that EGF can regulate phosphorus absorption in intestine. So, the present study would adopt an *in vitro* method (Ussing chamber system) to study the effect of EGF on phosphorus absorption.

The Ussing chamber system was first invented by Hans Ussing (Ussing and Zerahn, 1951), which has several components, including a circuit system, diffusion chambers, inserts, electrodes (Fig. 1), a data collection system, and a software support system (He *et al*., 2013). The Ussing chamber provides a physiologically relevant system for measuring the gastrointestinal epithelium permeability, gastrointestinal barrier function, and the transport of ions, nutrients and drugs across intestinal epithelial tissues (Maresca *et al*., 2002; Millar *et al*., 2002; Albin *et al*., 2007; Clarke, 2009; He *et al*., 2013). There are published studies involving the use of Ussing chambers to determine whether EGF affects glucose transport in the jejunum (Lennernäs *et al*., 1997; Millar *et al*., 2002), while, the study of EGF affects Pi transport using Ussing chamber is lacking. So, the aim of this study was to investigate the

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effect of EGF on phosphate absorption across intestinal mucosa of hens using the Ussing chamber system.

MATERIALS AND METHODS

Animals

All animal studies were approved by the Animal Care Committee of Guizhou Normal University (Guiyang, China). The experimental procedures were conducted in accordance with the Chinese guidelines for animal welfare.

Loman laying hens with similar weight (1.25±0.13 kg) were purchased from Hunan Institute of Animal Science and Veterinary Medicine (Changsha, China).

LDH activity determination

The release of LDH into the buffer can reflect the activity of isolated small intestine (Todd *et al*., 2016; Fadda *et al*., 2017; Tang *et al*., 2018a). To detect the LDH activity of isolated small intestine, four hens were sacrificed after 12 h of starvation. A 5 cm of duodenum (from distal of the gizzard to 1 cm distal of the bile duct), jejunum (1 cm distal from the bile duct to the Meckel's diverticulum), and ileum (Meckel's diverticulum to 5 cm proximal to the ileocecal junction) (Fang *et al*., 2012) were opened longitudinally, and immersed in a dish containing 20 mL Hepes-Tris buffer (6 g Hepes, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 8.18 g NaC1, adjust the pH to 7.4 using 1 M Tris), respectively. At 0 min, 30 min, 60 min, 120 min and 180 min, 0.5 mL buffer was collected to analysis LDH activity. The determination of LDH activity was used a Lacate Dehydrogenase Assay kit (No. A020-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer. Experiments were performed in four times.

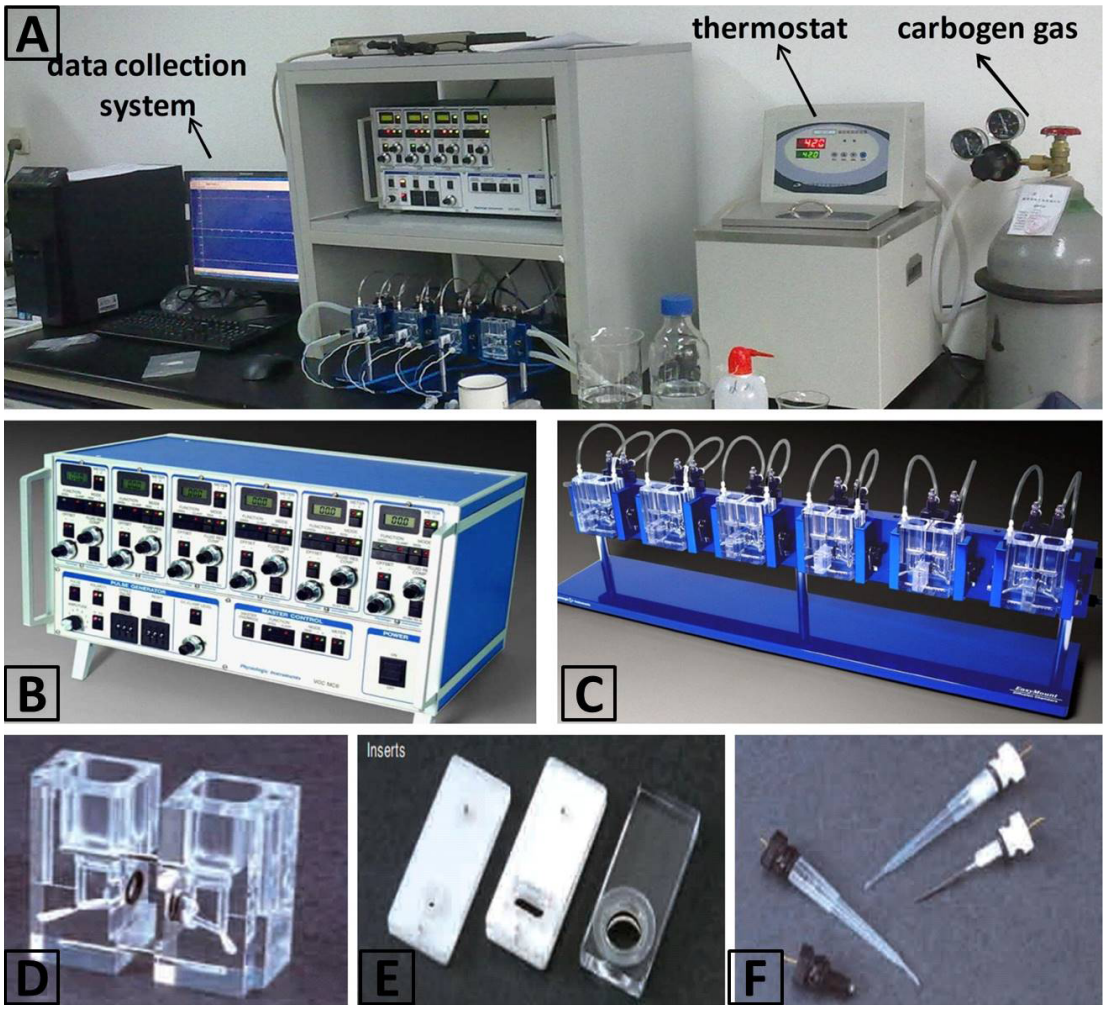

Fig. 1. The structure of Ussing chamber system. A, the panorama of the Ussing chamber; B, electronic circuitry (VCC-MC6); C, diffusion chamber (6 channels); D, diffusion pool and receiving room; E, inserts (P2313); F, electrodes.

Ussing chamber assay

A total of 20 Loman laying hens with similar weight (1.25±0.13 kg) were divided into 1 of 5 treatments: (i) Control group (0 ng/mL EGF), (ii) 50 ng/mL EGF group, (iii) 100 ng/mL EGF group, (iv) 150 ng/mL EGF group, and (v) 200 ng/mL EGF group. Each treatment had 4 replicates with one hen. All hens were fasted for 12 h before sacrificed. The duodenum, jejunum, and ileum were obtained immediately after slaughter for Ussing chamber studies. The Ussing chamber system (VCC MC6) was manufactured by Physiologic Instruments, Inc. (San Diego, CA, USA).

After the removal of muscle layers and serosal layer, the remaining mucosa samples were mounted between the two halves of Ussing chambers inserts (P2313) with an exposed serosal area of 0.71 cm^2. Mucosal compartments were filled with 4 mL Hepes-Tris buffer (pH 7.4) containing 0 ng/mL EGF (Peprotech, Rocky Hill, NJ, USA), 50 ng/mL EGF, 100 ng/mL EGF, 150 ng/mL EGF, 200 ng/mL EGF, respectively, and serosal compartments filled with 4 mL Hepes-Tris buffer (pH 7.4) simultaneously, circulated with carbogen gas (95% O_2, 5% CO_2) at a temperature of 37°C. After an equilibration period of 10 min, 1 mL of 0.35% Pi solutions was added into mucosal compartment, and 1 mL of 0.35% mannitol solution was added into serosal compartment. After 45 min of incubation, 0.5 mL of samples was collected from both compartments for Pi concentration analysis to calculate the absorption rate of Pi across intestinal mucosa. The determination of Pi concentration was use a Phosphate Assay Kit (No. C006, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer. During the experiment, TEER was monitored during the whole experimental period using a computer controlled voltage clamp device (Physiologic Instruments Inc., San Diego, CA, USA). Experiments were performed in four times. The absorption rate of Pi was calculated by following equations:

$$\text{Pi absorption rate (\%)} = \frac{(TP - SP - MP)}{TP}$$

Where TP is total Pi content, SP is the Pi content in serosal compartment, and MP is the Pi content in mucosal compartment.

Statistical analysis

All data were expressed as mean±standard deviation (SD). Data for single factor experiments were performed by one-way ANOVA procedure of SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA). Differences among treatment mean were determined using Duncan's multiple comparison test. Linear correlation analyses between EGF concentrations and TEER, and Pi absorption rate were performed by linear regression procedure of GraphPad Prism 7.0 software (Graph-pad Software Inc., San Diego, USA). $P < 0.05$ was considered significant.

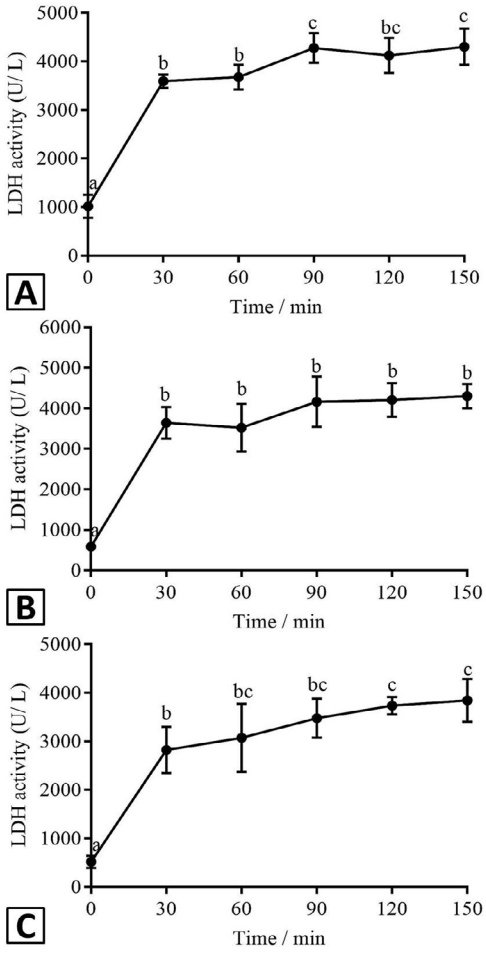

Fig. 2. The LDH activity of isolated small intestine. A, LDH activity of duodenum; B, LDH activity of jejunum; C, LDH activity of ileum. Data are represent as mean ± SD, n=4. a, b and c, means within error bars with different letters differ significantly ($P < 0.05$).

RESULTS AND DISCUSSION

The LDH activity of isolated small intestine

LDH is a stable glycolytic enzyme, when cells or organs suffered from stress, the cell membrane permeability would change, and LDH can be quickly released (Todd *et al*., 2016). So the release of LDH into the Hepes-Tris buffer can reflect the activity of isolated small intestine. As shown in Figure 2, the LDH activity of isolated duodenum (Fig. 2A), jejunum (Fig. 2B), and ileum (Fig. 2C) increased sharply during 30 min ($P < 0.05$). After 60 min, the LDH activity of isolated intestines reached a high levels, it

indicated that the activity of isolated intestines is weaken. So the Ussing chamber experiment should finished in 60 min. In the present study we incubated the isolated small intestine in chambers for 45 min.

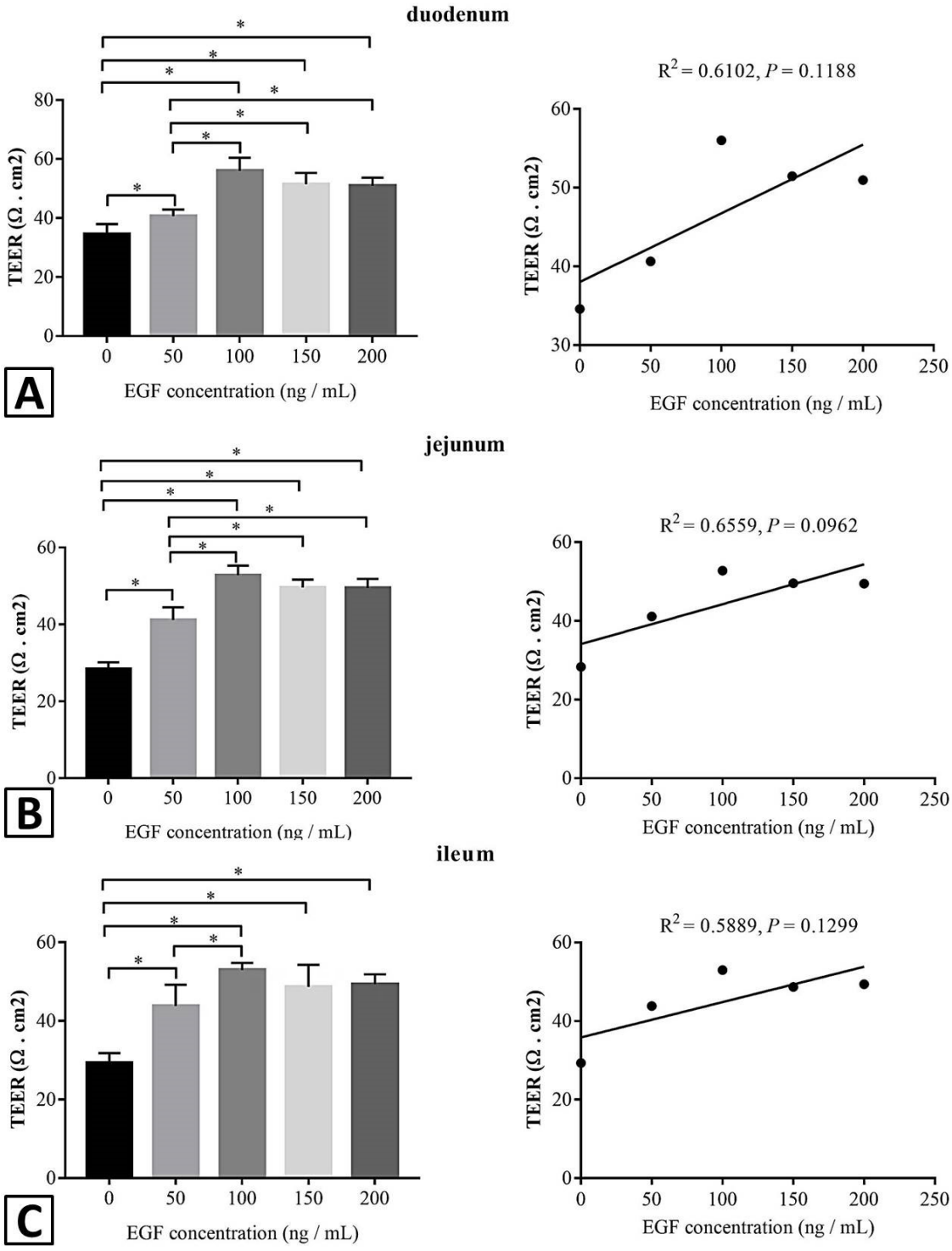

Fig. 3. Effect of epidermal growth factor on transepithelial resistance (TEER) of isolated small intestine. A, duodenum; B, jejunum; C, ileum. Data are represent as mean ± SD, n=4. * means $P < 0.05$.

Effects of EGF on TEER

The intestinal mucosal plays an essential role in the separation of the inside of the body from the outside environment (Oshima and Miwa, 2016). The integrity of intestinal is the foundation of nutrition absorption for animals, and can be evaluated through the measurement of the TEER (Garcia-Hernandez *et al*., 2015). TEER cans reflect the barrier function of intestine (Chen *et al*., 2015). Hence, the measurement of TEER has been mainly applied for assessing the permeability of tight junctions or the membrane perturbation by toxicants on intestinal epithelium (Chen *et al*., 2015). The Ussing chamber model is one of *in vitro* methods to measure TEER (Li *et al*., 2004; He *et al*., 2013). In the present study, the TEER of duodenum (Fig. 3A), jejunum (Fig. 3B) and ileum (Fig. 3C) treated with EGF (50, 100, 150, 200 ng/mL) both higher ($P < 0.05$) than that control group (none EGF); in duodenum (Fig. 3A) and jejunum (Fig. 3B), the 100, 150 and 200 ng/mL EGF group had a higher ($P < 0.05$) TEER than that 50 ng/mL EGF group; in ileum (Fig. 3C)

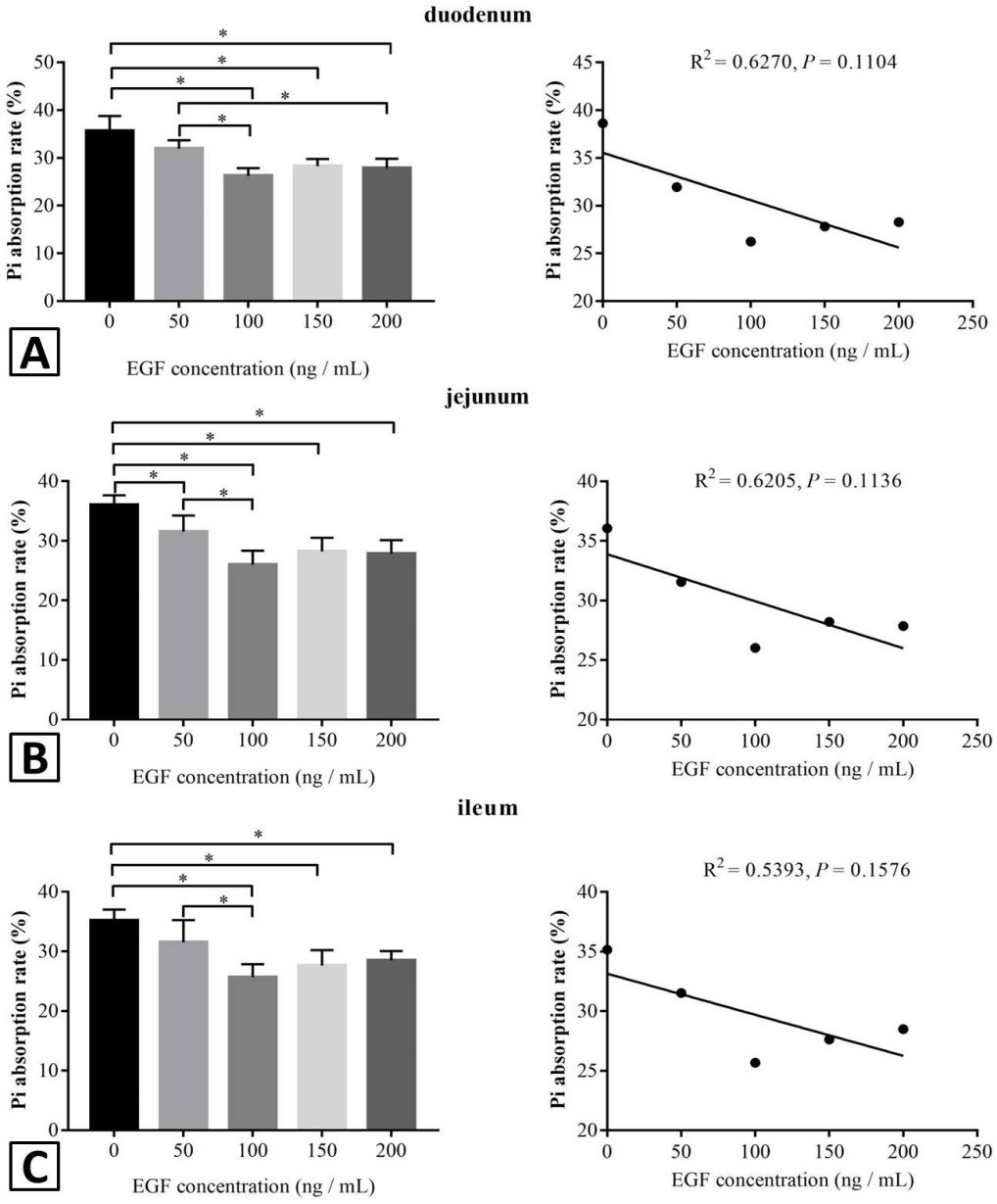

Fig. 4. Effect of epidermal growth factor on Pi absorption rate of isolated small intestine. A, Pi absorption rate of duodenum; B, Pi absorption rate of jejunum; C, Pi absorption rate of ileum. Data are represent as mean ± SD, n=4. * means $P < 0.05$.

100 ng/mL EGF group had a higher (P<0.05) TEER than that 50, 150 and 200 ng/mL EGF group. Both in duodenum (Fig. 3A), jejunum (Fig. 3B) and ileum (Fig. 3C) the EGF concentration had no liner relationship between EGF concentration and TEER (P>0.05). The results of present study indicated that EGF had a protective effect on the maintenance of intestinal integrity. Previous studies have demonstrated that EGF had the ability to increase TEER, and decrease epithelial paracellular permeability (Basuroy *et al*., 2006; Flores-Benitez *et al*., 2009; Garcia-Hernandez *et al*., 2015). The present study also confirmed that EGF can increase the TEER of isolated small intestines, which means that EGF plays an important role in maintaining intestinal integrity.

Effects of EGF on phosphate diffusion

Phosphorus is one of most important mineral element in life process (Fang *et al*., 2012; Giral *et al*., 2012). It is well know that Pi is mainly absorbed by simple diffusion and active absorption, and the active absorption of Pi is mainly mediated by NaPi-IIb (Forster *et al*., 2012; Xiang *et al*., 2012; Fang *et al*., 2012, 2016). Previous studies have demonstrated that EGF inhibited the expression of NaPi-IIb (Xu *et al*., 2001, 2003; Xing *et al*., 2017; Tang *et al*., 2018b). It means that EGF inhibited the active absorption of Pi in intestines. But whether EGF would promote simple diffusion of Pi is not know. The Ussing chamber technique is a simple, but powerful technique to investigate ion transport (Li *et al*., 2004). While, the study of EGF affects Pi transport using Ussing chamber is lacking. Hence, this study used Ussing chamber technique to study the effects of EGF on Pi absorption, to clarify the influence of EGF on phosphorus absorption. The results of Pi absorption rate was presented in Figure 4. It showed that the addition of EGF (50, 100, 150, 200 ng/mL) in Hepes-Tris buffer significantly (P<0.05) inhibited the diffusion of Pi from mucosal compartment to serosal compartment both in duodenum (Fig. 4A), jejunum (Fig. 4B) and ileum (Fig. 4C). However, both in duodenum (Fig. 4A), jejunum (Fig. 4B) and ileum (Fig. 4C) the EGF concentration had no liner relationship between EGF concentration and Pi absorption rate (P > 0.05). The reasons of EGF inhibited the diffusion of Pi may be interpreted from the following two aspects: firstly, EGF has trophic effects on intestine (Bedford *et al*., 2015; Xu *et al*., 2015). It can protect intestinal mucosa from exogenous injury to some extent in short times, which confirmed by the increase of TEER in the present study. So, the permeability of intestine was decreased, which resulted in a decreased Pi diffusion. Secondly, as previous studies showed, EGF inhibited the expression of NaPi-IIb (Xu *et al*., 2001, 2003; Xing *et al*., 2017; Tang *et al*., 2018b). It means that EGF inhibited the active absorption of Pi in intestines, which also resulted in a decreased Pi absorption rate.

CONCLUSION

In summary, the results of this research suggested that: (i) it's better finished in 60 min when used the Ussing chamber system to evaluate the absorption of nutrients in small intestine; (ii) EGF enhanced the TEER of intestinal mucosa and inhibited the diffusion of Pi.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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