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Effects of S-Methylmethionine Sulfonium Chloride on the Expression of Mucin 2 and Relevant Growth Factors in Piglet Jejunal Epithelial Cells

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ABSTRACT

Background: Intestinal health of livestock and poultry is the basis of their body health. Different expression of growth factors is important cause of intestinal dysfunction, which further induced the illness of livestock and poultry. S-Methylmethionine Sulfonium Chloride (SMMSC) is also the main metabolite of methionine in animals. However, there are few studies exploring the relationship between SMMSC and intestinal epithelial growth factors.

Methods: Piglet jejunal epithelial cells IPEC-J2 were treated with different concentrations of SMMSC at 0.1, 0.5 and 1 mM for 24 h respectively. Then real-time quantitative polymerase chain reaction (RT-qPCR) and western blotting (WB) were employed to detect the expression of Mucin-2 (MUC2), epidermal growth factor (EGF), transforming growth factor beta (TGF-β_1), glucagon-like peptide-2 (GLP-2) and insulin-like growth factor-1 (IGF-1).

Result: The results showed that 0.5 and 1 mM of SMMSC could stimulate the expression of MUC2, EGF, GLP-2 and IGF-1 while inhibit the expression of TGF-β_1 in both mRNA and protein level (p<0.05). It is of great significance to repair intestinal mucosa injury, maintain intestinal mucosa barrier and promote intestinal development.

Key words: Grow factors, Intestinal mucosal epithelium, IPEC-J2, MUC2, SMMSC.

INTRODUCTION

Intestinal health of livestock and poultry is the basis of their body health. Intestinal epithelial cell is an important part of intestinal epithelial mucus barrier. It is the first barrier against the invasion of intestinal pathogenic bacteria and substances in microenvironment. The damage of intestinal epithelial cells is one of the most important causes of intestinal dysfunction, which further induced the illness of livestock and poultry. Mucin-2 (MUC2) is one of the main components of intestinal epithelial mucus barrier. Changes in the quality and quantity of MUC2 are associated with a variety of intestinal diseases (Arike *et al.*, 2017; Johansson *et al.*, 2008). MUC2 is a secretory mucin involved in the formation of mucus and is mainly secreted by goblet cells. Reduced MUC2 expression in the intestinal mucosa, caused by various pathogenic microorganisms or toxic substances, will increase intestinal mucosal permeability, ultimately leading to the intestinal dysfunction or colorectal cancer (Kasprzak *et al.*, 2018; Liu *et al.*, 2020). Growth factors, mainly including epidermal growth factor (EGF), transforming growth factor beta (TGF-β_1), glucagon-like peptide-2 (GLP-2) and insulin-like growth factor-1 (IGF-1), play an extremely important role in regulating intestinal cell proliferation and apoptosis, maintaining intestinal mucosal integrity and immune response (Connor *et al.*, 2016; Frater *et al.*, 2018).

S-Methylmethionine Sulfonium Chloride (SMMSC) is an anti-ulcer agent, which is mainly used to treat ulcer and inflammation in clinical practice. It promotes the repair and healing of wound surface of gastrointestinal mucosa and improves gastrointestinal motor function. On the other hand, SMMSC is also the main metabolite of methionine in animals. It functions as an important methyl donor and regulates the body growth, metabolism and functions (Kim *et al.*, 2010;

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Lee *et al*., 2012). However, there are few studies exploring the relationship between SMMSC and intestinal epithelial growth factors. The present study investigated the effects of SMMSC on the mRNA and protein level of MUC2, EGF, TGF-β_1, GLP-2 and IGF-1 in IPEC-J2 cells. It laid a foundation for the application of SMMSC in livestock and poultry production.

MATERIALS AND METHODS

Experiment

All the experiments were carried out in Henan Key Laboratory of Unconventional Feed Resources Innovative Utilization, Henan University of Animal Husbandry and Economy. The experiments were carried out from 2020.02 to 2021.09.

Cell culture

IPEC-J2 cell was purchased from Zhengzhou Aibokang Technology Co., LTD. Cells were cultured in DMEM-F12 medium (Solarbio) with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Invitrogen). Cells were cultured under the condition of 37°C with 5% CO_2. When cells reached at logarithmic growth stage, 0.25% trypsin was used to digest cells and for further experiment.

IPEC-J2 cells in the logarithmic growth phase with good growth condition were seeded in a 96-well plate at a concentration of 5×10^3 cells/well. Then, the ordinary medium was changed in to the medium contains 0 mM (control group), 0.1 mM (low concentration group), 0.5 mM (medium concentration group) and 1 mM (high concentration group) SMMSC and further cultured for 24 h.

Reagents

Bicinchoninic acid (BCA) protein assay kit for protein detection was purchased from Beyotime Biotechnology. Antibodies against β-actin, MUC2, EGF, TGF-β_1 and IGF-1 were purchased from Abcam and antibody against GLP-2 was purchased from Bioss. Trizol was purchased from Beyotime Biotechnology.

RNA extraction and quantitative real time PCR (qRT-PCR)

IPEC-J2 cell were treated with different concentrations of SMMSC, then cells were washed with RNase free PBS and lysed with Trizol for mRNA extraction. The concentration and quality of mRNA were determined by a spectrophotometer and the OD260/OD280 ratio between 1.8 and 2.0 was supposed to meet the experimental requirements. Reverse transcription was carried as follow: Total RNA 3.15 μg, Oligo $(dT)_{18}$ (10 μM) 2 μL, dNTP (2.5 μM) 4 μL, 5 × Hiscript Buffer 4 μL, Hiscript Reverse Transcriptase 1 μL, Ribonuclease Inhibitor 0.5 μL and added ddH_2O to 20 μL for total volume. Then reaction was performed as follow: 25°C for 5 min, 50°C for 15 min, 85°C for 5 min and 4°C for 10 min. qRT-PCR was carried out as follow: cDNA 1 μg, Forward Primer (10 μM) 0.4 μL, Reverse Primer (10 μM) 0.4 μL, SYBR Green Master Mix 10 μL, 50 × ROX Reference Dye 0.4 μL, ddH_2O 4.8 μL. Then reaction was performed as follow: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 sec, annealing at 60°C for 60 sec, extension at 95°C for 15 sec, repeated for 40 cycles. Melting curve collection: 60°C for 60 sec, 95°C for 15 sec. Primers used in this study were shown in Table 1.

Western blot (WB)

Total 40 μg protein of each sample or 6 μL marker was added into the loading well. Followed with electrophoresis at constant pressure of 80 V and then changed to constant pressure of 120 V when bromophenol blue indicator reached the junction of concentrated and separated gel. The electrophoresis was stopped when bromophenol blue reached the bottom of gel. Then we transfer the protein to the PVDF membrane. The gel and membrane were clamped according to the order: black plate-fiber pad-filter paper-gel-PVDF membrane-filter paper-fiber pad-white plate. Next, we clamped the plate into the transfer instrument and transfer the protein at 200 mA for 120 min. PVDF membrane was blocked in TBST containing 5% skim milk powder at room temperature for 2 h. Then, the PVDF membrane was incubated with the primary antibody (1:1000 dilutions) overnight at 4°C. Followed with HRP labeled secondary antibodies at room temperature for 2 h and the signal were detected with ECL detection system and analyzed by BandScan.

Statistical analysis

The experimental data were statistically processed by Excel (2007). The statistical analyses were performed with the SPSS statistical package (24.0). One-way ANOVA and LSD method were used to compare differences between groups. Values were expressed as the mean±standard deviation (SD). $p<0.05$ was considering statistically significant differences. $P>0.05$ was considering no significant difference.

Table 1: Primers used in this study.

Gene	Primer	Sequence (5′-3′)	PCR products
β-actin	Forward	CTCCATCATGAAGTGCGACG	242 bp
	Reverse	CCTGCTTGCTGATCCACATC	
EGF	Forward	TGCTGGAGATGGAAACCTGT	231 bp
	Reverse	CAGGTGTAGTTTCCCTCCGT	
TGFβ-1	Forward	CAAGGTCCTGGCTCTGTACA	150 bp
	Reverse	CAGGAACGCACGATCATGTT	
GLP-2	Forward	CATTCACCAGCGACTACAGC	244 bp
	Reverse	ATGGCGACTTCTTCTGGGAA	
IGF-1	Forward	CTGTGCTTGCTCTCCTTCAC	230 bp
	Reverse	TTGAGGGGTGCACAGTACAT	
Muc-2	Forward	GCACACCACTGACCCCGACG	112 bp
	Reverse	GGACCCGAGGTTGACGAGCC	

RESULTS AND DISCUSSION

Effects of SMMSC on MUC2 mRNA and protein level in IPEC-J2 cells

As can be seen from Fig 1 and 2, the mRNA and protein level of MUC2 in the medium concentration and high concentration groups were significantly increased compared with the control group (p<0.05). The mRNA and protein level of MUC2 in the low concentration group were also increased, though it was not significant (p>0.05).

Effects of SMMSC on mRNA and protein level of EGF, TGF-β_1, GLP-2 and IGF-1 in IPEC-J2 cells

As shown in Fig 3, the mRNA and protein level of EGF and IGF-1 in the groups with SMMSC were significantly increased compared with the control group (p<0.05). The mRNA and protein level of TGF-β_1 in the groups with SMMSC were significantly decreased compared with the control group (p<0.05). The mRNA and protein level of GLP-2 in the medium concentration and high concentration groups but not low concentration group were significantly increased compared with the control group (p<0.05).

Intestinal tract is an important organ for digestion and nutrient absorption and recently was reported to maintain the stability of the internal environment because it works as an important barrier to protect the body from antigens, toxins and pathogens (Arrieta *et al.,* 2006). The integrity of intestinal structure and function is maintained by the regularly proliferation and differentiation of intestinal epithelial cells (Okamoto *et al.,* 2006). Malnutrition and environmental stress can easily lead to morphological and structural changes of intestinal mucosa, resulting in secretion disorder of cytokines and antibodies in intestinal mucosal immune system, leading to intestinal diseases of livestock and poultry. Intestinal diseases will affect both quantity and quality of livestock and poultry and bring significant economic losses. Therefore, how to use feed additives or other ways to improve the

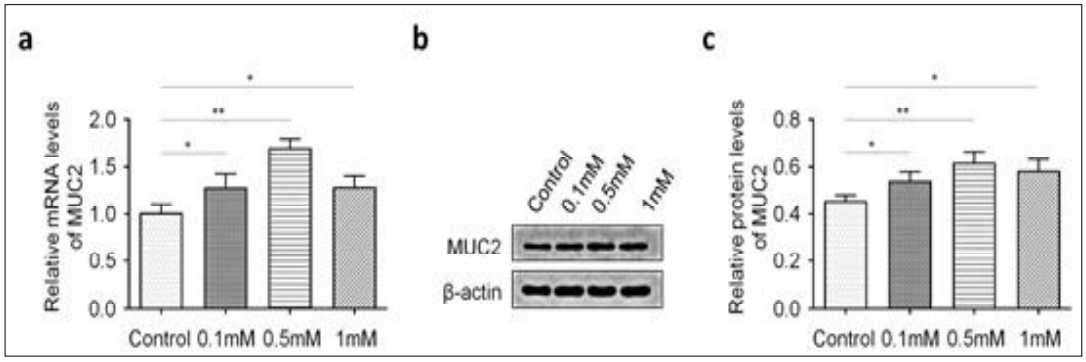

Fig 1: Effects of different concentrations of SMMCS on MUC2 in IPEC-J2 cells. (a): qRT-PCR was performed to detected effects of different SMMCS concentrations on MUC2 mRNA level. (b): WB was performed to detected effects of different SMMCS concentrations on MUC2 protein level. (c) Quantity of WB results. p<0.05 (*), p<0.01 (**), p<0.001 (***).

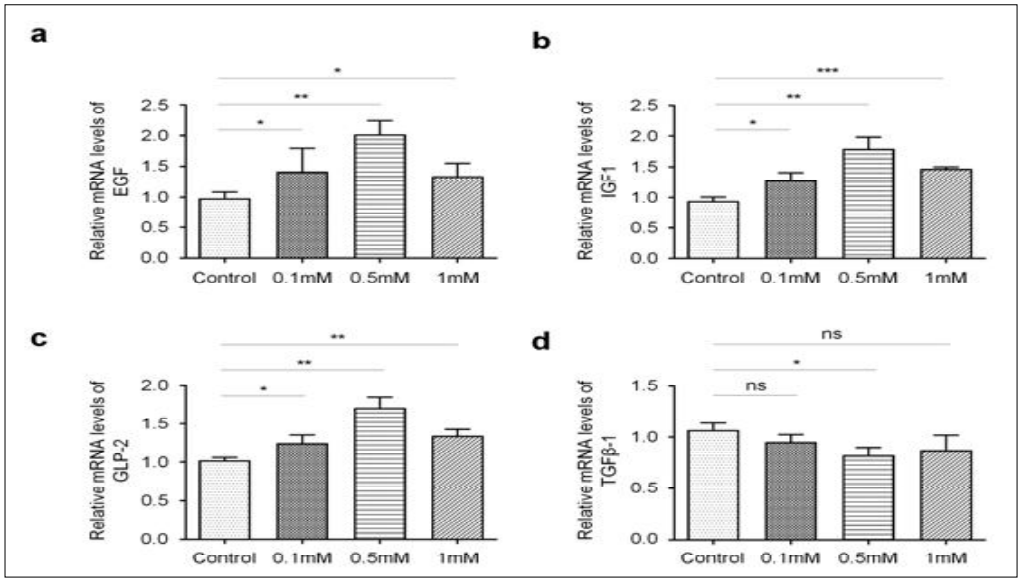

Fig 2: Effects of different concentrations of SMMCS on mRNA of growth factors in IPEC-J2 cells. qRT-PCR was performed to detected effects of different SMMCS concentrations on EGF (a), IGF-1 (b), GLP-2 (c) and TGF-β1 (d) mRNA level. p<0.05 (*), p<0.01 (**), p < 0.001 (***).

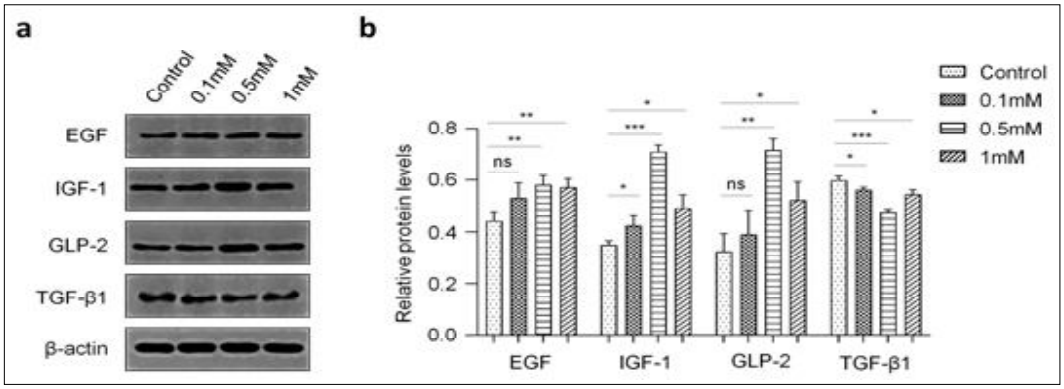

Fig 3: Effects of different concentrations of SMMCS on protein of growth factors in IPEC-J2 cells. (a): WB was performed to detected effects of different SMMCS concentrations on EGF, IGF-1, GLP-2 and TGF-β1 mRNA level. (b): Quantity of WB results. ns: Not significantly, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

intestinal health of young animals and to reduce intestinal diseases occurrence has become a focus for scholars.

Amino acids play an important role in regulating the balance of intestinal dynamic, maintaining intestinal health and preventing intestine from diseases (Gottardo *et al.* 2016). Sulfur-containing amino acids (SAA) plays essential role in the growth of livestock and poultry. Changes in dietary SAA will affect the metabolism, gene expression and function of intestinal cells (Tesseraud *et al.,* 2009). The one carbon metabolite of Methionine (Met), a kind of SAA, is S-adenosylmethionine (SAM). SAM is a key mediator to control intestinal metabolic homeostasis and it's also an important regulator of the activity of intestinal stem cells (ISCs) (Burgess *et al.,* 2014; Shoveller *et al.*, 2005; Tang *et al.*, 2017). Notably, SMMSC can also be synthesized from methionine and SAM and SAM plays an important role in protecting the integrity of intestinal epithelial structure and function (Yan *et al.,* 2018).

Decreased dietary Met or SAM has been reported to affect growth performance (Elango, 2020; Elshorbagy *et al.*, 2013; Jin *et al.*, 2007), lipid metabolism (Jha *et al.,* 2014) and translation ability of animals (Laxman *et al.,* 2013). Consistent with our previous report that SMMSC can promote the proliferation and reduce apoptosis of jejunal epithelial cells in piglets. We hypothesized that SMMSC may affect cell survival, proliferation and differentiation, growth and apoptosis by regulating epidermal growth factor (EGF), insulin-like growth factor (IGF), TGF-β_1 and glucagon-like peptide 2 (GLP2) (Arda-Pirincci and Bolkent, 2014; Houle *et al.*, 1997; Huang and Huang, 2005; Iwabu *et al.*, 2004; Massague *et al.*, 2000; Petersen *et al.*, 2003; Xu *et al.*, 2020). Thus, relevant experiments were carried out in this study.

Our results showed that different concentrations of SMMSC can significantly improve the expression of EGF and IGF-1 in both mRNA and protein level in IPEC-J2 cells. The expression of EGF and IGF-1 in IPEC-J2 cells were more significantly improved in the SMMSC concentration of 0.5 mM, indicating that 0.5 mM SMMSC functions better in changing the level of MUC2 and the growth factors. These results indicated that SMMSC play an important role in intestinal development and therefore affected the nutrient absorption, cell proliferation and intestinal repair in piglet jejunal epithelial cells. These were consistent with previous studies that SMMSC affected intestinal development via regulating EGF and IGF-1 (Kim *et al.*, 2010; Xu *et al.*, 1994). The role of SMMSC in intestinal development may due to its function as the methyl donor, regulating of SAM, increasing the activity of S-adenosyll homocysteine and stimulating the methylation process (Kim *et al.*, 2010; Lee *et al.*, 2012). However, the detail molecular mechanism needs to be further investigated.

Moreover, we found that 0.1 mM SMMSC had no significant effect on the expression of GLP-2 in IPEC-J2 cells while 0.5 and 1 mM SMMSC significantly increased the expression of GLP-2 in IPEC-J2 cells. These results indicated that SMMSC have anti-inflammatory and anti-injury function and can increase intestinal barrier function by regulating GLP-2 expression in a dose dependent manner, which was also consistent with previous findings (Brubaker, 2006; Yang *et al.*, 2021). TGF-β_1 was revealed to induce apoptosis of the intestinal cells (Hao *et al.*, 2019; Seoane and Gomis, 2017). Accordingly, the addition of 0.5 or 1 mM SMMSC significantly reduced the expression of TGF-β_1 in IPEC-J2 cells and therefore improved the activity of IPEC-J2 cells.

The integrity of the mucus barrier is the first line of gastrointestinal protection. Intestinal mucus layer plays a major role in preventing intestinal mechanical, chemical and biological attack and helps maintain the dynamic balance of the intestine (Cornick *et al.*, 2015; Etienne-Mesmin *et al.*, 2019).

Currently, the physical barrier role and immunomodulatory function of mucus barrier has attracted increasing attention of scientists. MUC2 is secreted by goblet cells of the small intestine and colon and it's a major component of intestinal mucus. MUC2 is secreted when goblet cells were stimulated by different hormones, neurotransmitters (*e.g.*, vasoactive intestinal peptide, acetylcholine) or lipids (*e.g.*, bile acid, prostaglandin, butyric acid) (Herath *et al.*, 2020; Kim and Ho, 2010; Yamashita and

Melo, 2018). Studies have shown that EGF promotes the secretion of MUC2 by stimulating the proliferation and differentiation of intestinal goblet cells Bedford *et al.* (2015). Butyrate was proved to change the expression of MUC2 in a dose-dependent manner Hatayama *et al.* (2007). Animal studies have shown that Threonine can increase the expression of MUC2 in small intestine of weaned piglets Wang *et al.* (2010). The present study showed that SMMSC could up-regulate the expression of MUC2 in IPEC-J2 cells in a dose-dependent manner and 0.5 mM SMMSC had the most significant effect on the mRNA and protein of MUC2 in IPEC-J2 cells. These results indicated that SMMSC could regulate MUC2 so as to affect intestinal mucosal barrier function.

CONCLUSION

The results showed that SMMSC could increase the transcription and expression of MUC2, EGF, GLP-2 and IGF-1 in IPEC-J2 cells and decrease the transcription and expression of TGF-β_1, it is of great significance to repair intestinal mucosa injury, maintain intestinal mucosa barrier and promote intestinal development.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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