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Effects of Methylmethionine Sulfonium Chloride on Activity and Tight Junction Protein Expression of Intestinal Porcine Jejunum Epithelial Cells (IPEC-J2)

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ABSTRACT

Background: The intestinal mucosal epithelium acts as a physical and biochemical barrier and plays an important role in regulating of barrier function and immune homeostasis. Methylmethionine sulfonium chloride (MMSC) is a multifaceted amino acid that is critical to the normal physiology of the gastrointestinal tract. The present study investigated the effects of extracellular MMSC on intestinal epithelial cell line (IPEC-J2).

Methods: IPEC-J2 cells were treated with 0.1, 0.5 and 1 mM MMSC, respectively for an additional 24 h. CCK-8 assay was used to evaluate cell proliferation. The cell Annexin V-FITC/PI apoptosis were analyzed by flow cytometry (FCM) method. The mRNA transcript and protein expression levels of tight junction proteins in IPEC-J2 cells were detected by real-time quantitative polymerase chain reaction (RT-qPCR) and western blotting (WB).

Result: The results showed that MMSC could stimulate IPEC-J2 cells proliferation and inhibit cell apoptosis. In addition, the RT-qPCR and WB results indicated that 0.5 mM MMSC significantly increased the mRNA and protein expression of tight junction proteins, including occludin, claudin-1 and zonula occludin-1 (Zo-1). These findings may provide valuable information to investigate further the possible mechanism and function of MMCS on the intestinal barrier function.

Key words: Intestinal mucosal epithelium, IPEC-J2, MMSC, Protein expression, Tight junction.

INTRODUCTION

The intestinal mucosal epithelial barrier serves as the first line of defense against the invasion of pathogenic microorganisms and toxic substances in the intestinal tract (Yu and Li 2014). It is formed by the interaction between the epithelial cells and the tight junction proteins, which play an important role in ion transport, selective absorption of essential nutrients, blockade of harmful substances and so on (Barrett 2008). Recently, it is reported that piglet diarrheal and intestinal inflammation is closed to the intestinal mucosal barrier and the function of tight junctions are important for the maintenance and repair of the intestinal mucosal barrier (Guevarra *et al*. 2018, Yang *et al*. 2019).

Tight junctions are intercellular adhesion complexes on epithelial cells, which was composed of integral membrane proteins as well as cytosolic adaptor proteins (Roehlen *et al*. 2020). Occludin, claudins and zonula occluden (Zo), the main transmembrane component of tight junctions proteins, have been implicated in regulating properties and functions of tight junctions (Suzuki and Hara 2009, Zong *et al*. 2019). Tight junction proteins were believed to be one of the most important components of the intestinal mucosal barrier and played an important role in health and disease (Farkas *et al*. 2012, Kojima *et al*. 2003). So far, intestinal porcine jejunum epithelial cells (IPEC-J2) line isolated from the mid-jejunum of a neonatal piglet is established as an in vitro model for porcine infection studies and nutritional studies (Liu *et al*. 2010, Zakrzewski *et al*. 2013).

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Methylmethionine sulfonium chloride (MMSC), generally known as vitamin U, which is a multifaceted amino acid and used as gastroprotective drug in treating injuries or ulcerations of the digestive tract and skin (Kim *et al*. 2015, Salim 1992). It is reported that MMSC promoted the mucus barrier of rat gastric mucosa, as it improved the amount and immunoreactivity of surface mucus cell-derived mucin (Ichikawa *et al*. 2009). More recently, studies showed that

MMSC pre-treatment in female rats might prevent valproic acid-induced renal damage and lung toxicity (Gezginci-Oktayoglu *et al*. 2016, Oztay *et al*. 2020). However, little is known about the effect of the MMSC on the intestinal epithelial cell barrier. Therefore, it would be of interest to examine whether supplementation of MMSC modulates the expression of tight junction proteins.

In the present, we aimed to explore the influence of MMSC on IPEC-J2 cells, including the cell viability, as well as the mRNA and protein expression levels of cell tight junction genes. Results showed MMSC displayed an effective activity in promoting the expressions of the key tight junction proteins. These findings laid a foundation to further study the effects of MMCS on intestinal permeability and barrier function in IPEC-J2 cells.

MATERIALS AND METHODS

IPEC-J2 cells culture

IPEC-J2 cells were cultured and maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM-F12, Solarbio) supplemented with 10% foetal bovine serum (FBS, Gibco) and penicillin–streptomycin (100 mg/ml; Invitrogen) at 37°C, 5% CO_2 and 95% relative humidity. When IPEC-J2 cells reached confluence, 0.25% trypsin was added for digestion and passage and then cells in the logarithmic growth phase were selected for experiments.

Infection of IPEC-J2 cells with MMSC

IPEC-J2 cells were cultured onto 96-well plates at 5×10^4 cells/plate or 6-well plates at 1×10^6 cells/plate and incubated in complete DMEM-F12 containing 10% FBS. After 24 h incubation, culture supernatant was discarded and cells were cultured in DMEM-F12 without FBS containing different concentrations of MMSC for an additional 24 h. Untreated cells were used as a negative control. IPEC-J2 cells in group 1 were infected with 0.1 mM MMSC, whereas group 2 and 3 were infected with 0.5 mM MMSC and 1 mM MMSC, respectively. IPEC-J2 cells in 96-well plates were used to estimate cell proliferation and in 6-well plates were collected for cell apoptosis and protein expression. Each sample was repeated with three times.

Cell proliferation analysis

CCK-8 assay was used to evaluate cell proliferation. Added 10 µL CCK-8 solution (Solarbio) to 96-well plates and continued to incubate for 4 h. Cell proliferation activity (%) was calculated as: mean OD450 nm of experimental cells / mean OD450 nm of negative control group cells.

Annexin V-FITC/PI apoptosis assay

IPEC-J2 cells in 6-well plates were collected, digested into single-cell suspensions with EDTA-free trypsin and then subjected to the Annexin V/PI Apoptosis Detection kit (Invitrogen) for staining according to the manufacturer's instructions. The stained cells were analyzed by flow cytometry (FCM) method.

RT-PCR detection of mRNA expression levels

IPEC-J2 cells treated with MMSC or untreated cells in 6-well plates were cultured for 24 h. Total RNA of the IPEC-J2 cells was extracted from the cells using Trizol® reagent (Invitrogen). RNA was reversely transcribed into cDNA using the TaKaRa reverse transcription kit (TaKaRa). Real-time polymerase chain reaction (RT-PCR) was performed by using FastStart Universal SYBR® Green Master (Rox) (Roche). RT-PCR amplification procedure was as follows: initial denaturation for 10 minutes at 95°C followed by 35 cycles, with each cycle consisting of 30 seconds at 95°C, 30 seconds at 60°C, in addition to a final extension for 5 minutes at 72°C. The amplification reactions with β-actin as internal reference and three pairs of target primers were listed in Table 1. The relative expression amount was calculated by using equation $2^{-\Delta\Delta CT}$(Livak and Schmittgen 2001). Each sample and negative controls (no template) were run in triplicate.

Western blotting detection of protein expression

Western blotting (WB) analysis was performed to evaluate the protein expression. Briefly, IPEC-J2 cells in 6-well plates treated with MMSC in 6-well plates were washed one time with PBS and then total proteins of IPEC-J2 cells were extracted by using the radioimmunoprecipitation (RIPA) lysis buffer (NCM Biotech) supplement with 1% protease inhibitor (Solarbio). 1 ml lysis buffer was added to the well, lysed for 30 minutes on ice, then collected and centrifuged at 14, 000 × g for 20 minutes. Supernatant fractions contained total proteins. The protein concentration of supernatant fractions was quantified by a standard bicinchoninic acid (BCA) protein assay (Pierce). An equal amount of protein was separated on SDS-PAGE gels and then transferred onto PVDF membranes. PVDF membranes were blocked with

Table 1: Sequence of primers used for detection of genus *Brucella*.

Gene name	Primer	Sequence (5′-3′)	PCR products
β-actin	Forward	CTCCATCATGAAGTGCGACG	242 bp
	Reverse	CCTGCTTGCTGATCCACATC	
Zo-1	Forward	ATCTCGGAAAAGTGCCAGGA	176 bp
	Reverse	CCTTCCCCTCAGAAACCCAT	
Claudin-1	Forward	TGGAAGATGATGAGGTGCAGA	180 bp
	Reverse	CAGTGAAGAGAGCCTGACCA	
Occludin	Forward	ACGGTTATGGCTATGGAGGG	193 bp
	Reverse	AACACCATCACACCCAGGAT	

5% non-fat dry milk in PBST at 37°C for 2 hours. After six times washing with PBST, membranes were incubated with primary antibody at 4°C overnight. The antibodies used in our study including rabbit polyclonal anti-Occludin (1:1000; Proteintech), rabbit polyclonal anti-Claudin-1 (1:1000; Proteintech), rabbit polyclonal anti-Zo-1 (1:1000; Proteintech), mouse anti-β actin (1:500; Boster). After six times washing with PBST, secondary antibody, such as anti-rabbit IgG, anti-mouse IgG (1:5000, Jackson ImmunoResearch), was added and incubated at 37°C for 30 minutes. Finally, membranes were examined with ECL reagent (NCM Biotech) followed by analysis using Gel Doc TM XR (Bio-rad). The intensity of the blotting was quantified using qualityone software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.00 (GraphPad Software). The comparison among groups was analyzed by one-way ANOVA. Data were shown as the mean ± SEM. Statistical significance was determined at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

RESULTS AND DISCUSSION

Cytotoxicity of MMCS on IPEC-J2 cells

To estimate cytotoxic effect of MMCS, CCK-8 assay was used to evaluate the IPEC-J2 cells proliferation. As shown in Table 2, compared with control group, the highest level of IPEC-J2 cells proliferation was detected in group 2 (0.5 mM), whereas no significant difference was observed between group 2 (0.5 mM) and group 3 (1 mM). Cells treated with three different concentrations of MMSC showed high cell viability, indicating that MMCS is nontoxic to IPEC-J2 cells.

Effects of MMSC on IPEC-J2 cells apoptosis

FCM method was performed to detect IPEC-J2 cells apoptosis. As shown in Fig 1, cell survival was highest in group 2 (0.5 mM), reaching to 97.02%, followed by 95.74% in group 3 (1 mM) and 94.75% in group 1 (0.1 mM), while 93.42% in control group. As shown in Table 3, MMCS would significantly reduce early and late cell apoptosis of IPEC-J2 cells ($p < 0.05$), comparing with untreated cells. There was no dose-dependent of MMCS on inhibition of IPEC-J2 cells apoptosis, but 0.5 mM MMCS showed best effect on inhibition of cells apoptosis. Detailed information was summarized in Table 3.

Analysis of mRNA expression levels of tight junction genes in IPEC-J2 cells

RT-PCR was conducted to determine the mRNA expression levels of tight junction genes, including occludin, claudin-1 and Zo-1. As shown in Fig 2a, the addition of 0.5 mM MMCS and 1 mM MMCS to the culture medium both significantly increased the mRNA expression of occludin in comparison with the mRNA expression in the control group. Interestingly, when IPEC-J2 cells were treated with 0.5 mM MMCS, the mRNA expression level of claudin-1 and Zo-1 would significantly improve ($p< 0.01$), but not observed in the group 3 (1 mM MMCS) (Fig 2b and Fig 2c). Moreover, when IPEC-J2 cells treated with 0.1 mM MMCS, the mRNA expression of these three tight junction genes showed no difference with those in control group (Fig 2). These results indicated that there was a dose-dependent effect of MMSC in stimulating mRNA expression and the 0.5 mM MMCS showed best effect on increasing the mRNA expression of tight junction genes.

Analysis of protein expression levels of tight junction genes in IPEC-J2 cells

For estimating effect of MMCS on protein expression levels, WB analysis was further used to evaluate tight junction proteins expression. As shown in Fig 3, in comparison with the control group, three different concentrations (0.1 mM, 0.5 mM and 1 mM) of MMSC significantly increased the protein expression of occludin ($p<0.01$, $p<0.0001$, $p<0.001$). The protein expression of claudin-1 significantly improved in 0.5 mM MMSC ($p<0.0001$) and 1 mM MMSC ($p<0.001$). However, only 0.5 mM MMSC was added into the medium, the relative protein abundance of Zo-1 significantly improved ($p<0.001$), but not observed in the 0.1 mM MMSC and 1 mM MMSC group. These results implied that MMCS supplemented may stimulate tight junction proteins expression and the 0.5 mM MMCS exhibited a higher level of protein expression.

Table 2: Effects of different concentrations of MMCS on IPEC-J2 cells proliferation.

	Control	0.1 mM	0.5 mM	1 mM
Cell proliferation activity (%)	$91.91±6.25^{c}$	$107.30±7.29^{b}$	$118.7±1.87^{a}$	$112.77±13.12^{ab}$

Note: Results were presented as means ± SEM of triplicate observations. Different lowercase letters in the same column denoted statistically significant difference ($p < 0.05$) and the same letters indicated no significant difference between the two groups ($p > 0.05$).

Table 3: Results of MMCS on cell cycle of IPEC-J2 cells.

	Control	0.1 mM	0.5 mM	1 mM
Late cell apoptosis (%)	$2.28±0.13^{a}$	$1.67±0.08^{b}$	$0.69±0.05^{c}$	$1.13±0.11^{d}$
Early cell apoptosis (%)	$4.09±0.19^{a}$	$3.20±0.08^{b}$	$2.13±0.11^{c}$	$2.62±0.15^{d}$

Note: Results were presented as means ± SEM of triplicate observations. Different lowercase letters in the same column denoted statistically significant difference ($p < 0.05$) and the same letters indicated no significant difference between the two groups ($p > 0.05$).

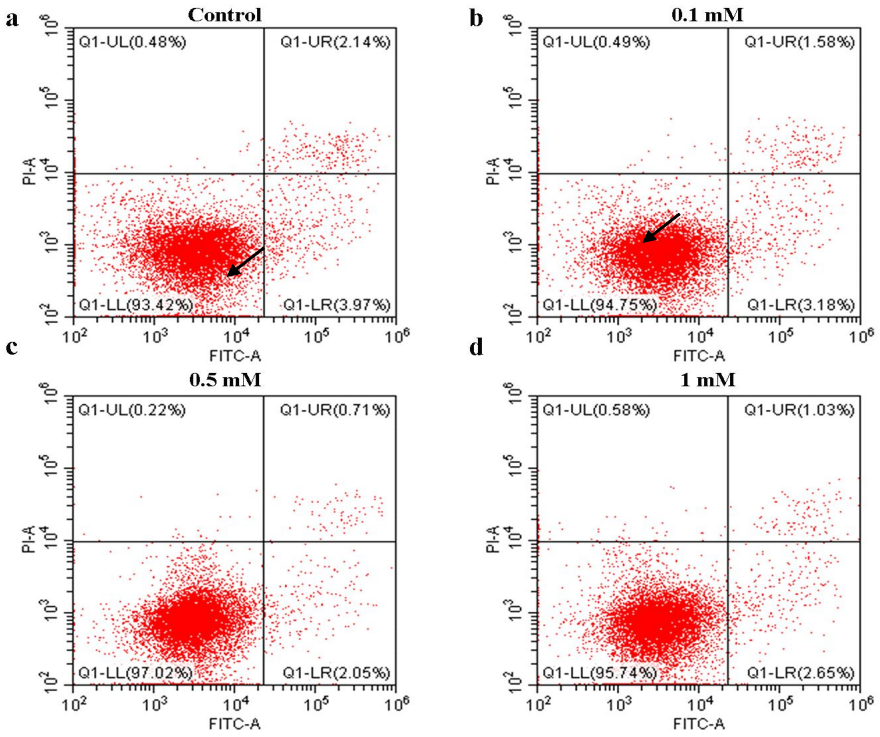

Fig 1: Effects of different concentrations of MMCS on IPEC-J2 cells apoptosis.

IPEC-J2 cells treated with MMCS in 6-well plates were collected, stained with the Annexin V/PI Apoptosis Detection kit and analyzed by FCM. Untreated IPEC-J2 cells were used as the control. a untreated IPEC-J2 cells; b IPEC-J2 cells treated with 0.1 mM MMCS; c IPEC-J2 cells treated with 0.5 mM MMCS; d IPEC-J2 cells treated with 1 mM MMCS. UL region was merornecrosis (upper left), LL region was cell survival (lower left), UR region was late cell apoptosis (upper right) and LR region was early cell apoptosis (lower right).

Intestinal microbiology and health of the mammals play an important role in the digestion and absorption of nutrients, growth of animals, improve immunity and disease resistance and so on (Li *et al*. 2019, Yatsunenko *et al*. 2012). In recent years, along with the application of antibiotics abuse, intestinal health problems in livestock and poultry are becoming increasingly serious, impacting the production performance and economic benefits of animal husbandry industry. Intestinal permeability was closely to prevent harmful substances from invading and important in animal subjects with diarrheal disease (Boudry *et al*. 2004). Therefore, there has been an increasing scientific interest on the use of nutritional manipulations or other mechanisms to improve the intestinal barrier function of young animals.

Amino acids or trace elements are indispensable for intestinal health and biological functions, which affected the intestinal mucosal barrier by regulating the expression of intestinal cytokines and tight junction proteins (Tretola *et al*. 2020, Wu 2009, Xia *et al*. 2016). Occludin, claudin-1 and Zo-1 were considered to be the key proteins of tight junction integrity and controlled the function and permeability of the intestinal (Brun *et al*. 2007). It is reported that Zn supplementation in dietary treatments would reduce intestinal permeability by enhancing occludin and Zo-1 expression (Sturniolo *et al*. 2002, Zhang and Guo 2009). Reducing arginine concentration was feasible in IPEC-J2 cells, which guaranteed nutrient uptake and cell barrier function by improving the occludin and claudin-1 expression (Xia *et al*. 2016). Moreover, studies showed that added dietary threonine would benefit to growth performance, health, immunity and gastrointestinal function of weaning pigs (Trevisi *et al*. 2015).

In the present study, different concentrations of MMSC were chosen to evaluate its effect on activity and tight junction protein expression of IPEC-2 cells. As shown in Table 2 and Table 3, MMCS is nontoxic to IPEC-J2 cells and cells treated with three different concentrations of MMSC showed high cell viability and low cells apoptosis (Fig.1). As tight junction protein and adherent junction are important in the maintenance of epithelial integrity, we further analyzed mRNA expression and protein expression of tight junction protein in IPEC-J2 cells. Results from RT-PCR showed that when IPEC-2 cells treated with 0.5 mM MMSC, mRNA levels of tight junction genes, including occludin, claudin-1 and Zo-1 were significantly increased ($P<0.001$, $P<0.01$ and $P<0.01$) (Fig 2). Whereas higher MMSC supplemented (1 mM), there were no differences compared to the control group. Similarly, this phenomenon existed in protein expression levels of tight junction proteins. As shown in Fig. 3, 0.5 mM MMSC significantly enhanced protein expression of occludin, claudin-1 and Zo-1. Excepting Zo-1, 1 mM MMSC treated with IPEC-J2 cells, would stimulate occludin,

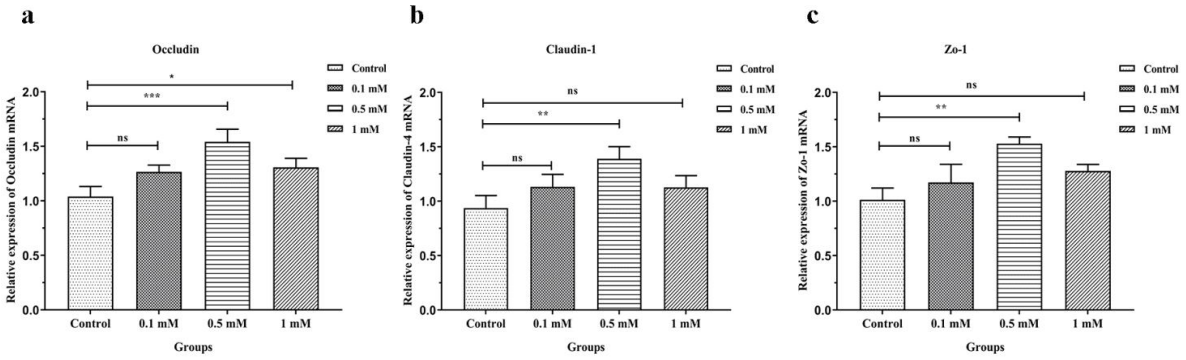

Fig 2: RT-PCR results of mRNA expression of tight junction genes.

IPEC-J2 cells in 6-well plates were extracted using Trizol® reagent and RT-PCR was used to analyze the mRNA expression levels of tight junction genes. a Occludin; b Claudin-1; c Zo-1. Relative gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method and β-actin was used as internal reference. The data above were representative of three independent experiments and error bars indicated the SEM (n=3). Statistical significance was determined at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

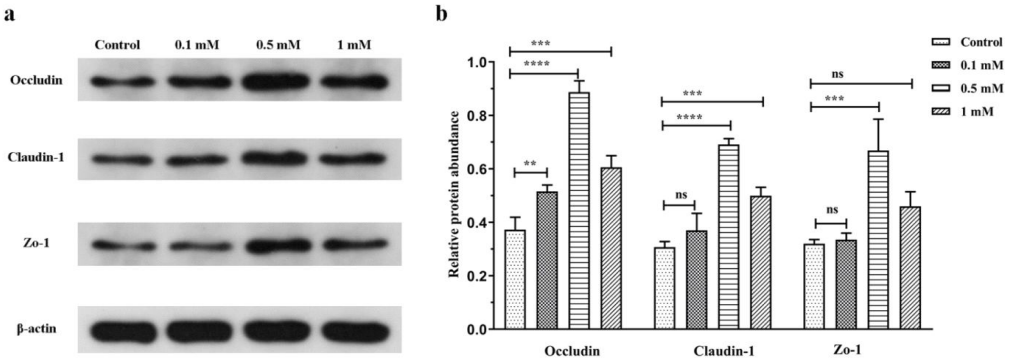

Fig 3: Effects of different MMCS concentrations on tight junction proteins expression.

a Results from WB anaysis; b Relative protein expression levels of tight junction proteins. The value of protein expression=densitometry units of target protein/densitometry units of β-actin protein detected by WB. Error bars indicated the SEM (n=3) and statistical significance was determined at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

claudin-1 protein expression. These data above demonstrated that MMCS, within the suitable concentration, could enhance occludin, claudin-1 and Zo-1 expression in IPEC-2 cells. It indicated that MMCS may play a role in regulating intestinal permeability and barrier function of intestinal mucosal epithelial cells.

In summary, MMCS is nontoxic to IPEC-J2 cells and has a function in promoting the mRNA and proteins expressions of the key tight junction proteins (occludin, claudin-1 and Zo-1). We found that 0.5 mM MMCS exhibited a higher level of mRNA and protein expression in IPEC-J2 cells.

CONCLUSION

The present study investigated the effects of extracellular MMSC on intestinal epithelial cell line (IPEC-J2). The results showed that MMSC could stimulate IPEC-J2 cells proliferation and inhibit cell apoptosis. In addition, the real-time quantitative polymerase chain reaction (RT-PCR) and western blotting (WB) results indicated that 0.5 mM MMSC significantly increased the mRNA and protein expression of tight junction genes, including occludin, claudin-1 and zonula occludin-1 (Zo-1). These findings may provide valuable information to further investigate the possible mechanism and function of MMCS on the intestinal barrier function.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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