Chlorogenic acid enhances intestinal barrier by decreasing MLCK expression and promoting dynamic distribution of tight junction proteins in colitic rats

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ABSTRACT

The aim of the present study was to evaluate the effect of chlorogenic acid (CGA) on the mucosal barrier function and tight junction proteins in colitic rats. CGA (60 mg/kg body weight) significantly decreased the ratio of lactulose to mannitol (L/M) (P < 0.05), and endotoxin level (P < 0.01) in CGA + colitis group, compared to colitis group. CGA supplementation in CGA + colitis group increased the expression of occludin (P < 0.05), claudin-1 (P < 0.01), ZO-1 (P < 0.05), decreased the expression of myosin light chain kinase (MLCK), compared to colitis group. CGA also increased the phosphorylation level of occludin (P < 0.01) at tight junction, and decreased the phosphorylation level of occludin (P < 0.05) and claudin-1 (P < 0.05) on the basolateral membranes. These results indicate that CGA supplementation attenuates colonic barrier damage by decreasing MLCK expression and promoting molecular dynamic distribution of tight junction proteins in colitic rats.

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Keywords:
Chlorogenic acid
Intestinal barrier
Tight junction
Myosin light chain kinase
Dynamic distribution
Colitis

1. Introduction

Chlorogenic acid (CGA) is one of the most abundant phenolic acids and is widespread in fruits, vegetables, cereal, tubers crop (Clifford, 1999), having multidirectional biological activity. Numerous scientific studies have confirmed the antioxidant, anti-inflammatory, and regulating intestinal flora activities of CGA. Recently, the effect of phenolic acid on intestinal barrier attracts the attention of many researchers.
The mucosa is the largest contact area and one of the most important barriers against the outside environment. Under normal conditions, the physical barrier allows the transport of essential dietary nutrients and electrolytes from intestinal lumen into the circulation and prevents the passage of unwanted or harmful substances, thereby maintaining the intracellular homeostasis (Yang et al., 2015). Defects in barrier integrity play a crucial role in the pathogenesis of various diseases, such as inflammatory bowel disease or irritative bowel disease (Antoni, Nuding, Wehkamp, & Stange, 2014). The structural basis of intestinal barrier is cell junctions between intestinal epithelial cells. These junctions include tight junction (TJs), adhesion junction and gap junction (Ma et al., 2013). Among these components, the TJs constitute the major determinant of the intestinal physical barrier. TJs are protein complexes composed of integral membrane proteins, cytoplasmic plaque proteins, and cytoskeletal proteins. The most important integral components of the TJs include occludin, claudins, and zonula occludens-1 (ZO-1) (Ulluwishewa et al., 2011).

Accumulating evidence have indicated that the change of TJs is triggered by the phosphorylation of myosin light chain (MLC), which predominantly depends on myosin light chain kinase (MLCK) activation (Turner, 2009). The activated MLCK catalyses the MLC phosphorylation, which in turn results in the slip and depolymerisation of F-actin. Clayburgh et al. (2005) revealed that epithelial MLCK is essential for intestinal barrier dysfunction and that this barrier dysfunction is critical to pathogenesis of diarrhoeal disease. Turner (2006) proved that targeted inhibition of MLCK can affect this cytoskeleton dependent TJ dysfunction.

CGA can inhibit inflammatory cell infiltration, notably neutrophil recruitment into lung and inhibit inflammatory cytokines release (Li et al., 2014). Recent studies found that CGA regulated the antioxidant activities against ischaemia and reperfusion injury in the small intestine of rat (Sato et al., 2011). Furthermore, the studies in vitro showed that ferulic acid increased zonula occludens-1 (ZO-1) and claudin-4 transcription in T84 colon cells (Bergmann, Rogoll, Scheppach, Melcher, & Richling, 2009). Our previous study has found that CGA decreased intestinal permeability and increased the expression of intestinal tight junction proteins (occludin and ZO-1) in weaned rats challenged with LPS (Ruan et al., 2014). And other researchers also found the similar phenomenon that phenolic acid could decrease intestinal permeability and increase intestinal barrier function (Grilli et al., 2015; He et al., 2016). However, the effect of CGA on the phosphorylation of tight junction proteins, polymerisation of F-actin and MLCK kinase and its specific mechanisms were not clear.

According to the evidence, we make the hypothesis that CGA inhibits the activation of MLC kinase and promotes the phosphorylation of tight junction proteins, thereby ameliorating intestinal epithelial tight junction damage. The structure and the distribution of tight junction, MLCK, F-actin and tight junction-related proteins were measured to verify the hypothesis.

2. Material and methods

2.1. Animal care

Female Sprague-Dawley rats (aged 6 weeks, weighing 189.67 ± 2.88 g) were obtained from Changsha Tianqin Biotechnology Co. (Changsha, Hunan, China). All procedures were approved by the Animal Care and Use Committee of Nanchang University.

All animals were fed in a specific pathogen-free condition, temperature-controlled atmosphere (22 ± 1 °C at 40–60% relative humidity) under a 12 h light per 12 h dark cycle in Animal Laboratory of Jiangxi Province Center for Disease Control and Prevention (Nanchang, Jiangxi, China). All animals were allowed to acclimate to their environment for 1 week before the experiment. The experimental diets were formulated to meet the nutrient requirements conforming to China General Quality Standards for Animal Feed (GB14924.1-2001) (Table 1) (Xu et al., 2013). All rats were anaesthetised with pentobarbital after a specified protocol.

2.2. Introduction of colitis and experimental design

After a week of adaptation period, an ulcerative colitic model was induced by previously described methods (Qiu et al., 2011)

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**Table 1 – The composition and nutrient of experiment diet.**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content (%)</th>
<th>Chemical composition</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>14.0</td>
<td>Digestible energy (Mal/kg)</td>
<td>3.40</td>
</tr>
<tr>
<td>Corn</td>
<td>43.0</td>
<td>Crude protein (%)</td>
<td>21.0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>24.0</td>
<td>Crude fat (%)</td>
<td>4.5</td>
</tr>
<tr>
<td>Full fat soybean extruded</td>
<td>8.0</td>
<td>Calcium (%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1.4</td>
<td>Total phosphate (%)</td>
<td>0.7</td>
</tr>
<tr>
<td>Whey powder</td>
<td>3.0</td>
<td>Sodium (%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Fish meal</td>
<td>3.2</td>
<td>Met + Cys (%)</td>
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</tr>
<tr>
<td>Limestone</td>
<td>1.3</td>
<td>Lys (%)</td>
<td>1.35</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.1</td>
<td>Thr (%)</td>
<td>0.88</td>
</tr>
<tr>
<td>Vitamin-mineral premix*</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The nutrient levels of the diets were based on China General Quality Standards for Animal Feed (GB14924.1-2001).

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and our published paper (Ruan et al., 2014). Rats were deprived of food but not water for 24 h before induction of colitis. Fasted animals were lightly anaesthetised with 1% pentobarbital sodium. One millilitre of trinitro-benzene-sulphonic acid (TNBS) with a dose of 100 mg/kg body weight was dissolved in 50% ethanol (v/v). The TNBS solution was introduced into the proximal (8 cm) to the anus through the plastic catheter. Following administration of TNBS, the animals were kept in a head-down position for 60 s to allow the samples to move through the gastrointestinal tract.

All animals were distributed in four experimental groups: Control group, CGA group, colitis group, and CGA + colitis group. CGA was purchased with 98% purity (Aladdin, Shanghai, China) and dissolved in sterile saline. CGA supplement (60 mg/kg body weight per day) was intragastrically administered into the rats in CGA group and CGA + colitis group for 28 days between 09:00 am and 10:00 am. The control group and colitis group were intragastrically administrated with the same volume of sterile saline.

At the end of the experimental period and after a fasting period of 12 h, the animals were sacrificed by cardiac exsanguination under anaesthesia with an intraperitoneal injection of 10% potassium chloride. Blood samples, and intestine were dissected, weighted and immediately frozen.

2.3. Measurements and sample collection

After the rats were euthanised, blood samples, gut tissues (jejunum, ileum and colon), livers, kidneys, and spleens were harvested. Serum samples were prepared after centrifugation of blood samples for 10 min at 10,000 rpm at 4 °C, and immediately frozen and kept at −80 °C. The gut tissues were washed with ice-cold 0.9% saline solution to remove visible mucus and dietary debris. Subsequently, gut tissues were either fixed in 10% buffered formalin, embedded in paraffin or snap-frozen in liquid N2, and stored at −80 °C until further use. Liver, kidneys, and spleens were washed with ice-cold 0.9% saline solution and weighed.

2.4. Lactulose-mannitol test for intestinal permeability

Intestinal permeability was assessed by the lactulose-mannitol test (Ruan et al., 2014). A pretest sample of urine was collected after 6 h of fasting to measure the baseline sugar in the urinary tract. After the pretest sample was obtained, rats were orally given 100 mg of lactulose and 50 mg of mannitol dissolved in 2 mL of water. Urine was collected for 6 h and stored at −20 °C until assay. The concentration of lactulose and mannitol in urine were measured by the high-performance liquid chromatography (HPLC) method. Then, the L/M excretion ratios (L/M ratio) were calculated.

2.5. Measurement of endotoxin for intestinal permeability

The intestinal permeability with the indicator of endotoxin was determined with the method of Keshavarzian et al. (2009). Blood was collected from rats at the time of sacrifice. It was centrifuged for 10 minutes under 4000 × g in order to separate erythrocyte and serum. Serum samples were then analysed for endotoxin by kit.

2.6. Transmission electron microscopy of tight junction and desmosome

Microstructure of TJ was measured by transmission electron microscopy (TEM) according to that described method (Sileri et al., 2002). Colon samples were removed and placed in 2.5% glutaraldehyde. Then it was washed in 0.1 mol/L phosphate buffer at pH 7.2 for 2 h. Specimens were then rinsed in the same buffer and post-fixed in 1% osmium tetroxide for 2 h and dehydrated in graded acetones (30%, 50%, 70% with 2% uranyl-acetate, 90%, and 100%), cleared in propylene oxide and embedded in araldite. Semi-thin sections were cut with a diamond knife and stained lightly with 1% toluidine blue. Ultrathin sections (0.8 μm) were then cut with a diamond knife, stained with uranyl acetate and lead citrate, and observed by TEM (JEM-1200, JEOL Ltd, Tokyo, Japan).

2.7. Immunohistochemistry analysis for tight junction proteins

Colon samples were removed and fixed in 10% buffered formalin, embedded in paraffin and cut into 5 μm-thick sections (Bressebet et al., 2013). Sections were deparaffinised and rehydrated respectively with dimethylbenzene and alcohol (75%, 95%, and 100% alcohol). Then they were incubated in 3% H2O2 for 10 min, retrieval antigen with microwave, washed in PBS, and incubated with goat serum albumin for 20 min. Sections were incubated with rabbit anti-occludin (1:100, Invitrogen, Carlsbad, CA, USA), rabbit anti-claudin-1 (1:100, BS-0790R, Bios, Beijing, China), or rabbit anti-zonulin-1 (1:100, BS-1329R, Bios, Beijing, China) at 37 °C for 2.5 h and were washed with PBS. Biotinylated secondary antibodies were polyclonal swine anti-rabbit. Sections were incubated with dianaminobenzidine (DAB) for 5 min, counterstained with haematoxylin, and the reaction was stopped in distilled water. At last, the sections were dehydrated (with 75%, 95%, and 100% alcohol), cleared (with dimethylbenzene), and permanently mounted (with neutral resin size). The sections were observed under XD-202 inverted biological microscope at 400× magnification with ISCapure 3.0 (Jiangnan Novel Optics Co. Ltd, Nanjing, Jiangsu, China).

2.8. Immunofluorescence analysis for MLCK and F-actin

Immunostaining was executed as Guttmann et al. (2006) described. Colonic tissues (0.5–1 cm) were frozen using liquid nitrogen and saved in a −80 °C refrigerator. Then the colon tissues were made into frozen sections and cut into 30 μm sections. Sections were washed with PBS and incubated with 10% normal goat serum for 1 h at 25 °C. The samples were incubated with primary antibody (rabbit anti-MLCK, 1:100) overnight at 4 °C. Sections were washed and incubated with secondary antibody. After washing with PBS, FITC-Phalloidin (1:100) was used to stain the sections at 25 °C for 1 h. After washing with PBS, the cover slips were mounted using glycerinum. Images were obtained using Ti-DH Nikon microscope (Nikon, Yokohama, Japan) at 100× magnification.
2.9. Western blot analysis of MLCK and TJ protein

TJ proteins (occludin and claudin-1) were extracted by Nonidet P-40 (NP-40) lysis buffer (25 mmol/L HEPES/NaOH, pH 7.4, 150 mmol/L NaCl, 4 mmol/L EDTA, 25 mmol/L NaF, 1% NP-40, 1 mmol/L Na$_3$VO$_4$, and protease inhibitors) for the soluble or insoluble fraction. MLCK was extracted by SDS lysis buffer (25 mmol/L HEPES/NaOH, pH 7.5, 4 mmol/L EDTA, 25 mmol/L NaF, 1% SDS, and 1 mmol/L Na$_3$VO$_4$).

Western blot analysis was carried out as described previously (Oshima, Koseki, Chen, Matsumoto, & Miwa, 2011). The protein content on the lysates was estimated using the Coomassie brilliant blue method. The proteins (80 μg) were then loaded onto SDS-PAGE gels (occludin with 10% SDS-PAGE gels, claudin-1 with 8% SDS-PAGE gels, and MLCK with SDS-PAGE gels). After electrophoresis, the proteins were transferred to PVDF membranes (2 h, 200 mA). The membranes were blocked in 5% BSA for 1 h at 25 °C. After blocking, the membranes were exposed to rabbit anti-claudin-1 antibody, rabbit anti-claudin-1, or rabbit anti-MLCK antibody overnight at 4 °C. The membranes were washed with TBS-T buffer and exposed to horseradish peroxidase-labelled goat anti-rabbit IgG antibody. The membranes were again washed with TBS-T buffer and treated with western blotting detection reagent. Next, the intensity of each band was scanned and quantified using image processing software.

2.10. Statistical analysis

All data are presented as mean ± SE. Differences between variants were analysed by one-way ANOVA and the Tukey multiple comparison test using the statistical package of SPSS 11.0 (SPSS Inc., Chicago, USA). Values of P < 0.05 were regarded as statistical significance.

3. Result

3.1. Intestinal permeability

The levels of endotoxin in the blood and the ratio of L/M in urine are the important symbols to reflect intestinal permeability. There was no difference in the urinary L/M ratios between control group and CGA group. The urinary L/M ratio in colitis group was significantly increased compared with control group (p < 0.01). CGA supplementation in colitis rats significantly decreased the urinary L/M ratio compared with colitis group (p < 0.01) (Fig. 1a).

Endotoxin was assessed in serum obtained from blood at sacrifice. The level of endotoxin in colitis group was significantly increased compared with control group (P < 0.01). CGA supplementation in colitis rats significantly decreased the endotoxin level compared with colitis group (P < 0.05) (Fig. 1b). The urinary L/M ratio had significant correlation with serum endotoxin values (r = 0.608, P < 0.01) (Fig. 1c).

3.2. Ultrastructure of tight junction and desmosome

TJs were located at the apical side of intestinal epithelial cell by the TEM method and images (Fig. 2). At the location of TJs, the membranes were in close proximity, appearing to fuse at the apical side. Desmosomes were also visible below the TJs. In control group and CGA group, the TJs appeared as typical membrane fusions with intact TJ structure and desmosomes (Fig. 2a and b). In contrast, TJ ultrastructure in colitis group was altered and TJs were discontinuous with few membrane fusions apparent in colon tissues, desmosomal integrity was impaired (Fig. 2c). The density of desmosomes in colitis group was decreased, indicating the disruption in TJ morphology. In CGA + colitis group, TJs appeared to have an intact structure.

![Fig. 1 – Effect of CGA on intestinal permeability of colon. (a) Urinary L/M ratio of rats; (b) serum endotoxin level in rats; (c) the correlation of urinary L/M ratio and serum endotoxin level. *P < 0.05, **P < 0.01, compared with control group; #P < 0.05, ##P < 0.01, compared with colitis group.](image-url)
The density of desmosomes was increased compared with colitis group (Fig. 2d). The results showed that CGA supplementation alleviated the distortion of TJs and desmosomes.

We also found that the microvilli were neatly and tightly arranged in control and CGA group. The microvilli became rarification and transformed into a partially collapsed state in colitis group. Meanwhile, the microvilli of CGA + colitis group were recovered compared with colitis group.

3.3. Distribution and expression of the TJ protein

The distribution and expression of the occludin, claudin-1, and ZO-1 were assessed with immunohistochemistry (Fig. 3a–i). TJ proteins were presented at the apical intercellular borders in a belt-like manner, encircling the cells and delineating the cellular borders. The tan or brown labelling of sections was the positive expression of TJ protein.

In control group, the expression of TJ-associated proteins (occludin, claudin-1, and ZO-1) was mainly observed in the basal epithelial layers and was the strongest labeling (Fig. 3a, e, i). The labelling level and location of TJ proteins of CGA group was the same as control group (Fig. 3b, f, j). Little interpretable labelling was observed in colitis group (Fig. 3c, g, k) in the epithelium. However, in CGA + colitis group, intense labelling in the epithelium was obtained compared to colitis group (Fig. 3d, h, l).

The expression of TJ-associated proteins (occludin, claudin-1, and ZO-1) was built according to the integrated optical density. The expressions of TJ-associated proteins (occludin, claudin-1, and ZO-1) in CGA group had no significant difference compared with control group. In the colitis group, expression of occludin was reduced markedly compared with control group (P < 0.05); similarly, protein abundance of claudin-1 was also significantly diminished compared with control group (P < 0.01). The reduction in the expression of claudin-1 and occludin (the main transcellular constituents of TJ) was accompanied by marked reduction of the main cytosolic plaque protein, ZO-1 compared to the control group (P < 0.01). However, the expression of occludin, claudin-1, and ZO-1 in CGA + colitis group was aggrandised significantly compared to colitis group (P < 0.05, P < 0.01, P < 0.05).

3.4. Western blot of tight junctions (occludin and claudin-1)

The expression of phosphorylated occludin and claudin-1 and its non-phosphorylation level were measured with western blot (Fig. 4). The levels of phosphorylated occludin (P < 0.01) and claudin-1 in CGA + colitis group increased significantly, compared to colitis group (Fig. 4a–c). The levels of non-phosphorylated occludin (P < 0.05) and claudin-1 (P < 0.05) in CGA + colitis group decreased significantly, compared to colitis group (Fig. 4d–f). The phosphorylation levels of occludin (P < 0.01) and claudin-1 (P < 0.05) in the NP-40-insoluble fraction in colitis group were decreased significantly compared to control group. Also the levels of non-phosphorylated occludin (P < 0.01) and claudin-1 (P < 0.05) on the basolateral membranes (in the NP-40-soluble fraction) were increased significantly as compared with control group.

3.5. Distribution of MLCK and F-actin

The distribution of MLCK and F-actin was examined (Fig. 5a–l). Immunofluorescent labeling with antisera specific for MLCK (Fig. 5a and d) showed that MLCKs were distributed in the villus tip in control group and CGA group. In colitis group, the expression of MLCK was enhanced and distributed in all the villus compared to control group (Fig. 5a and g). Nevertheless, the expression of MLCK in CGA + colitis group was lessened severely compared to colitis group (Fig. 5g and j). The F-actin staining showed a continuous clear line at the membrane of the epithelial cells in control group and CGA group (Fig. 5b and e). However, the F-actin of colitis group was localised at the whole villus, with some punctate staining in the cytoplasm (Fig. 5h). And the expression of F-actin in colitis group was tremendously decreased compared with control group (Fig. 5b and h). In CGA + colitis group, the expression of F-actin was enhanced compared with colitis group, and distributed as a line at the membrane of the epithelial cells (Fig. 5h and k).

3.6. Western blot of MLCK

A significant increase of MLCK expression was induced in colitis group when compared with control group (P < 0.01) (Fig. 6).
Fig. 3 – Effect of CGA on the expression of tight junction proteins (occludin, claudin-1, and ZO-1) in the mucosa of colon. (a–d) Immunohistochemistry of occludin of representative mucosa of colon; (e–h) immunohistochemistry of claudin-1 of representative mucosa of colon; (i–l) immunohistochemistry of ZO-1 of representative mucosa of colon; (m) statistical evaluation of effect of addition of CGA on the expression of occludin in the mucosa of colon; (n) statistical evaluation of effect of addition of CGA on the expression of claudin-1 in the mucosa of colon; (o) statistical evaluation of effect of addition of CGA on the expression of ZO-1 in the mucosa of colon. *P < 0.05, **P < 0.01, compared with control group; #P < 0.05, ##P < 0.01, compared with colitis group.
4. Discussion

In this study, we demonstrated that CGA supplementation could partly attenuate colonic epithelial barrier injury in colitic rats. The L/M and endotoxin assay revealed that CGA reduced the increased intestinal permeability in colitis. CGA reduced the increased colonic permeability and prevented the expression and location of occludin, claudin-1, and ZO-1. CGA can also inhibit the activation of MLCK and the depolymerisation of F-actin. And we also draw a conclusion that CGA may regulate the colonic barrier function through inhibiting MLCK pathway. These findings strongly suggest that CGA treatment should be viewed as a potential novel therapeutics for colonic barrier dysfunction in colitic rats. This is the first study reporting the cellular molecular mechanisms of chlorogenic acid regulating mucosal barrier and tight junction protein, which may be beneficial for guiding the functional food design and innovation.

Phosphorylation of TJ proteins has been linked to both TJ permeability and regulating TJ integrity (Ulluwishewa et al., 2011). Non- or less phosphorylated occludin is distributed on the basolateral membranes and that highly phosphorylated occludin is selectively concentrated at tight junctions as the NP-40-insoluble form (Sakakibara, Furuse, Saitou, Ando-Akatsuka, & Tsukita, 1997). Assembly and disassembly of tight junctions are associated with reversible phosphorylation of TJ proteins on Ser and Thr residues, indicating that Ser/Thr-phosphorylation of TJ proteins plays a crucial role in the regulation of tight junction integrity (Rao, 2009). These results suggested that the phosphorylation of occludin is a key step in tight junction assembly. Genistein and PP2 (Tyrosine kinase inhibitors) prevented lipopolysaccharide-induced de-phosphorylation of occludin on Thr residues (Sheth, Santos, Seth, Larusso, & Rao, 2007). Alcohol combined with burn injury resulted in a significant decrease in occludin and claudin-1 phosphorylation in small intestine compared to either alcohol or burn injury alone (Li, Akhtar, & Choudhry, 2012). Claudin-1 enhances the barrier function tight junctions via phosphorylation and subsequent integration into tight junctions, and threonine residue 203 of claudin-1 is required to enhance the barrier function of claudin-1-based tight junctions (Fujibe et al., 2004). In the presence of CGP77675 (nonreceptor tyrosine kinase inhibitor) after Ca<sup>2+</sup> repletion, occludin tyrosine phosphorylation was completely abolished and both tight junction formation and the increase of the TER were inhibited (Chen, Lu, Goodenough, & Jeansson, 2002). In our research, CGA...
significantly increased the phosphorylation of occludin and claudin-1 (in NP-40 insoluble components) in tight junction from colitic rats, and decreased the non-phosphorylation of occludin and claudin-1 in basolateral membranes (in NP-40 soluble components) (Figs. 3 and 4). These results prove that CGA can increase the phosphorylation of occludin and claudin-1, regulate dynamic distribution of occludin and claudin-1, thereby promoting the colonic barrier functions.

F-actin bundles were connected with TJ (Madara, 1987). The actin cytoskeleton plays several major functions at epithelial junctions (Ivanov, 2008). It was shown that the perijunctional F-actin ring is a dynamic structure which undergoes continuous F-actin depolymerisation and repolymerisation (‘treadmilling’) (Sandbichler, Egg, Schwerte, & Pelster, 2011). Gagat’s study indicated that structural stabilisation of F-actin, by overexpression of tropomyosin-1, preserved cell to cell interactions through the attenuation of cortical actin organisation into thin fibers; structural stabilisation of thin cortical F-actin fibers increases link between TJ proteins and actin cytoskeleton (Gagat et al., 2016). Wang et al. found that anillin (including

![Image of immunofluorescence images of MLCK (red) and F-actin (green) in the mucosa of colon.](705_Journal_of_Functional_Foods_26_(2016)_698–708)

**Fig. 5 – Effect of CGA on the colitis-induced disorder in MLCK and F-actin localisation.** (a, d, g, and j) The immunofluorescence images of MLCK (red) in the mucosa of colon; (b, e, h, and k) the immunofluorescence images of F-actin (green) in the mucosa of colon; (c, f, i, and l) the immunofluorescence merge images of MLCK (red) and F-actin (green) in the mucosa of colon. The arrows in a, d, g, and j point the expression of MLCK, the arrows in b, e, h, and k point the expression of F-actin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
actin filaments and myosin motors) knockdown activated c-Jun N-terminal kinase (JNK), and JNK inhibition restored AJ and TJ integrity and cytoskeletal organisation in anillin-depleted cells (Wang, Chadha, Feygin, & Ivanov, 2015). In our study, CGA increases the expression of F-actin (Fig. 5). It indicated that CGA affects the rearrangements of F-actin to relieve the colonic barrier dysfunction.

Contraction in the actomyosin ring are largely regulated by phosphorylation of myosin II regulatory light chain (MLC) by MLCK (Ulluwishewa et al., 2011). Inhibition of MLCK can decrease intestinal permeability and enhance intestinal barrier (Scott, Meddings, Kirk, Lees-Miller, & Buret, 2002). Detailed analysis of tight junction structure in intestinal epithelia monolayers demonstrated that the reorganisation of perijunctional actin, occludin, and ZO-1 depended on MLCK (Shen et al., 2006). 1, 25-dihydroxyvitamin D directly suppressed long MLCK expression by attenuating NF-kB activation, attenuated the increase in mucosal barrier permeability, and inhibited long MLCK induction and MLC phosphorylation (Du et al., 2015). Cao et al. revealed that berberine inhibits MLC phosphorylation mediated by MLCK and HIF-1α, which might be the molecular mechanism involved in the protective action of berberine against intestinal epithelial barrier dysfunction (Cao, Wang, Sun, He, & Wang, 2013). MLCK inhibition may ultimately provide a viable approach in restoring tight junction barrier function and preventing or treating intestinal disease (Cunningham & Turner, 2012). CGA supplementation significantly reduced MLCK expression compared with colitis group (Figs. 5 and 6), and F-actin expression has obviously risen (Fig. 5). The phosphorylation of occludin and claudin-1 has obviously increased in CGA + colitis group (Fig. 4a–c). According to our results, CGA repairs the colonic barrier distribution in colitis partially via inhibiting the activation of MLCK.

Does the protective effect of CGA contribute to CGA and its metabolites in enteric cavity? As chlorogenic acid is the ester of caffeic acid and quinic acid, Olthof, Hollman, and Katan (2001) revealed that one third of chlorogenic acid and almost all of the caffeic acid were absorbed in the small intestine of humans. Therefore, we speculate that absorbed chlorogenic acid, absorbed caffeic acid and quinic acid which were hydrolysed from chlorogenic acid in the intestine, collectively make a contribution in increasing intestinal barrier.

On the other hand, there might be protein–phenolic complexation/association effects between CGA and proteins. CGA did not contain any protein component as CGA was 98% pure. Before administration of CGA into the oral cavity, there are no protein–phenolic complexation/association effects. In the intestine, CGA may or may not be non-covalently combined with protein. CGA supplement (60 mg/kg body weight per day) means that a rat ingests 12–18 mg CGA everyday, which is a low content in feed. Prigent et al. (2003) found that the non-covalent interactions between protein and CGA do not have pronounced effects on the functional properties of globular proteins in food systems. In fact, it is unclear whether there are phenolic–protein complexation/association effects in the enteric cavity. The related research contents should be investigated in further studies.

In conclusion, our results demonstrate that CGA can decrease intestinal permeability and attenuate intestinal barrier damage. CGA can inhibit MLCK activation to prevent the MLC phosphorylation, and also increase the polymerisation of the F-actin in cytoplasm. CGA decreases the non-phosphorylation of occludin and claudin-1 to promote assembly of epithelial tight junctions in colitis rats. However, the specific mechanism of CGA on MLCK warrants further investigation.

Competing interests

The authors have no conflicts of interest to declare.

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