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Mast cell activation, TLR4-NF-κB/TNF-α pathway variation in rats' intestinal ischemia-reperfusion injury and Tongxinluo's therapeutic effect

Zhang Junxiu[1], Feng Yu[1], Hu Yanyan[2], Zhang Yin[1], Liu Yi[1], Yang Minghui[1] and Li Shaodan[1]*
[1]Institute of Traditional Chinese Medicine (TCM), Chinese People's Liberation Army General Hospital (PLAGH), Beijing, China
[2]First Affiliated Hospital of PLA General Hospital, Beijing, China.

Abstract: This study was designed to investigate mast cell activation and related TLR4-NF-κB/TNF-α pathway variation in 3 and 7 days' rats intestinal I/R injury, and TXL's intervention effect. Rat intestine I/R injury was carried out using superior mesenteric artery occlusion model with 30 min ischemia followed 3 or 7 days' reperfusion. Rats were administered TXL ultrafine power of 0.4, 0.8 and 1.6g/kg/d respectively for 3 or 7 days after modeling. Mast cell activation was determined by immunofluorescent double staining. TLR4, ANGPTL4 and microRNA126 were determined by RT-PCR. PECAM-1, NF-κB p65, TNF-α and VE-Cadherin were determined by immunohistochemical staining. Intestine I/R induced massively mast cell activation and overexpressed TLR4, NF-κB, TNF-α, PECAM-1, miR126 in 3 and 7 days. VE-cadherin and ANGPTL4 expression was reduced. TXL treatment attenuated mast cell activation and inhibited TLR4, NF-κB, TNF-α, PECAM-1 over-expression in 3 and 7 days, protected VE-cadherin and ANGPTL4 protein. Inflammation boomed in rats' intestine I/R injury for 3 and 7 days, characterized by mast cell and related TLR4-NF-κB/TNF-α pathway activation, accompanied with endothelial barrier dysfunction and enhanced vascular permeability. TXL treatment attenuated inflammation, protected endothelial barrier function. TXL treat intestine I/R injury, according with "Treat different diseases with the same method" in TCM theory.

Keywords: Intestine, I/R injury, mast cell, inflammation, traditional Chinese Medicine.

INTRODUCTION

Intestine is easily prone to ischemia-reperfusion (I/R) injury (Granger *et al*., 1986; Yamamoto *et al*., 2001). Intestine I/R injury could be found in various diseases, and regards as an important pathological process in diseases like intestine obstruction, severe infections, trauma, ischemic shock, cardiopulmonary dysfunction. Intestine I/R injury leads to severe inflammation which damage tissues and organs (Li *et al*., 2014). Different hypotheses are associated with intestine I/R injury, but a majority of studies share the opinion that inflammatory response is an essential pathogenesis in intestine I/R injury (Li *et al*., 2014; Jing *et al*., 2014). Inflammation response lead to tissue damage and remote organ injury (Wang *et al*., 2015). Most studies investigate inflammatory response in 24 hours after intestine I/R injury (Wu *et al*., 2014; Ji *et al*., 2015). I/R injury is a persistently chronic pathological process. It is great significance to investigate chronic intestine I/R pathological process.

Studies indicated that stimulation of Toll-like receptor 4 (TLR4) on macrophages induces secretion of multiple cytokines including Tumor necrosis factor-α (TNF-α), Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Interleukin-12 (IL-12) (McCuedy *et al*., 2001; Supajatura *et al*., 2001). Those cytokines cause smooth muscle contraction, capillaries expansion, increased capillaries permeability and other cytokines secretion increase, which lead to inflammation expansion and cell necrosis. Several reports suggest that mast cell was closely involved in inflammatory response and tissue damage in intestine I/R injury (Andoh *et al*., 2001). Mast cell activation and degranulation induce secretion of multiple cytokines, which participate in inflammatory response in intestine I/R injury. In addition, stimulation of TLR4 on macrophages induces TNF-α, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) secretion is one of the key mechanism of inflammation. This study is designed to explore mast cell degranulation and related TLR4-NF-κB/TNF-α pathway variation in inflammatory response of 3 and 7 days' rats intestinal I/R injury. In addition, changes of endothelial barrier and adhesion molecules are also investigated.

Tongxinluo (TXL), a compound prescription in dried superfine powder form, is prescribed based on the collateral disease doctrine from Traditional Chinese Medicine. The 12 ingredients and its proportion of TXL are exhibited in table 1. TXL has been permitted for clinical application by Chinese government since 1996 (state medical license NO. Z20060322). Component analysis experiments found that the primary chemical constituents of TXL were ginsenoside Rg1, ginsenoside Rb1, peoniflorin, jujuboside A, jujuboside B, isoborneol, and borneol (Su *et al*., 2010; Chen *et al*., 2009; Zhang *et al*., 2009). TXL treatment on patients with diseases

**Corresponding author:* e-mail: zjx6373120@126.com

associated with heart, brain ischemic reperfusion turns out effective in clinical research in China (Pan *et al*., 2012). Experiment studies of myocardial I/R injury have indicated TXL inhibiting inflammatory reaction and protecting endothelial structure, function against I/R injury (Cheng, 2017; Liu *et al*., 2014). "Treat different diseases with the same method" is one of clinical rule in TCM. Based on this theory, we investigate inflammatory response in 3 and 7 days intestine I/R injury and TXL's intervention effect.

MATERIALS AND METHODS

Drug preparation

TXL ultra fine powder (≤10μm) made of 12 traditional Chinese medicine (see table 1.) was purchased from Yiling Pharmaceutical co. (Shijiazhuang, Hebei, China). The quality of TXL ultra fine powder was strictly keep to quality management as described. We use physiological saline solution as solvent for dissolution of TXL ultra fine powder.

Animal preparation

One hundred male Sprague-Dawley rats of specific pathogen free (SPF) grade, weighing (200±20) g, were supplied by Animal Center of PLA General Hospital (Beijing, SCXK 2012-0001). Rats were raised in laboratory animal room and feed by professional breeder in Animal Center of PLA General Hospital. All animals were handled according to the criterions of Animal Research Committee of PLA General Hospital. Operative steps and experimental protocol were permitted by Animal Ethics Committee of PLA General Hospital.

Intestine ischemia/reperfusion injury

Rats were anesthetized with 0.3% (1ml/100g) pentobarbital sodium (Sigma, St. Louis, MO, USA) by intramuscular injection. Making a 2-3cm length incision in the middle of rats' abdomen. At the fourth mesentery windows ahead ileocecus, bluntly separate superior mesenteric artery. Place an artery clamp right ahead the fourth mesentery windows to block bloodstream for 30 min, and then loosen the clamp to induce reperfusion (Zhang *et al*., 2015). Close abdominal wall, aseptic suture of abdominal cavity. The rat intestine I/R injury model was established.

Experimental protocol

Rats of Sham group and I/R group, saline solution was given through intragastric at volume of 4 ml/kg/d for 7days (once a day). In the TXL+ I/R groups (TXL-L, TXL-M, TXL-H), saline solution in which dissolved TXL ultrafine powder was given through intragastric at volume of 4 ml/kg/d, equal to TXL ultrafine power dosage of 0.4, 0.8, and 1.6g/kg/d (TXL-L) respectively for 3 or 7 days' administration (once a day). First administration was carried out in the day after model establishment for rats in each group. Random number table method was used to divide rats into 5 groups, Sham, I/R, TXL-L(0.4g/kg + I/R), TXL-M (0.8g/kg + I/R), and TXL-H (1.6g/kg + I/R) groups (20 rats each, 10 rats for 3 day injury and 10 rats for 7 day injury in each group). Rats in each group were sacrificed by cervical vertebra resection at 3 or 7 days after model establishment. Fresh I/R injury intestine sections was collected 4 hours after last intragastric administration. The tissues were fixed in 4% buffered formalin or quick-freeze in -80 (Beijing, Dingguochangsheng biotech Inc). Fresh intestine tissue was embedded in paraffin.

Immunofluorescent double staining

Using c-Fos+Trytase immunofluorescent double staining to determine mast cell activation (Trytase is a neutral protease of mast cell, a marker of mast cell. c-Fos is an immediate reaction protein in mast cell gene transcription process, a marker of mast cell activation (Lewin *et al*., 1996; Metcalfe *et al*., 1997). The conventional paraffin embedded sections were cut 5μm thick slices every 1mm interval at each transverse section. After deparaffinization, pretreated intestine material using citric acid buffer in microwave for 1 min. Using 10% donkey to block intestine material for 30 min, then reacted with primary antibody overnight at 4°C. The next step antibody, anti-goat gG-Cy3 antibody (Thermo A10521) was diluted to 1:200 and incubated for 60min. Incubated intestine material first reacted with fluorescein labeled c-Fos, then used trypsin to stain nucleus. Eventually, washing the sides with TBS, then using Fluorescent Mounting Medium to mount. Using Fluorescence Microscope (Olympus BX43) to determine co-expression of c-Fos and Trytase. Activated green light of c-Fos (Abcam Ab83691) at 494nm, activated red light of Trytase (Abcam Ab15860) at 550nm. Image analysis using Image-Pro Plus, calculating IOD value.

Immunohistochemical staining

Immunohistochemistry for Platelet endothelial cell adhesion molecule-1 (PECAM-1), NF-κB p65, TNF-α and vascular endothelial-cadherin (VE-Cadherin). Antigens were unmasked by microwaving section in 10mmol/L citrare buffer, pH 6.0 for 15 min. Intestine material were placed in processing cassettes, dehydrated through a serial alcohol gradient. Then, pre-treated intestine material with 0.03% hydrogen peroxide methanol solution followed by immersing in a 10mM citric acid buffer under 6.0 pH situation, after that, place the intestine material in autoclave at 121°C for 5 min. Anti mouse-HRP (Beijing Kangweishiji Inc, Cw0102) which was diluted to 1:1000 reacted at 4°C overnight. The next step antibody, anti-rabbit-HRP (Beijing Kangweishiji Inc, Cw0103) was allowed to react, then washed with PBS followed by color development with DAB. Image analysis using Image-Pro Plus for image analysis, calculated the Integral optical density (IOD) value of PECAM-1 (Abcam Ab2736), NF-κB p65 (Abcam

AB32536), TNF-α (Abcam Ab16768), VE-Cadherin (Abcam Ab15560) in intestine I/R injury tissue.

Real-time fluorescence quantitative polymerase chain reaction

Total RNA was extracted with Trizol (Life Technologies Corporation, Cat# 15596018) from the placenta and total mRNA reverse transcription reaction was performed using PolyA plus tail method. Reverse transcription reaction system for TLR4 and Angiopoietin-1 (ANGPTL-1): 1μL 50μm Olifod (T), 6μg RNA Template, 10ul RNase-Freewater. Reverse transcription reaction system for miR126: 1μL o.2μm U6-RT, 1μL 0.2μm miR126, 3μg RNA Template, 10ul RNase-Free water. Then, the mix liquid was incubated for 10min at 70°C, ice bath for 2min, short centrifugation, continue to add 2X RT buffer, incubated for 50min at 42°C. After the reaction, keep 70°C for 10 min. Use Real Master Mix (SYBR Green, with Rox, Cat# 151105P1105H) as quantitative fluorescence PCR reaction system, experimental operation according to product specifications. Amplification procedures are: 95°C 5min, (95°C 15s, 60°C 15s, 72°C 15s) × 45 cycles. RealTime reaction system: 5μl Real Master Mix (2×), 0.5 ROX, 0.2μl upstream primer (10μM), 0.2μl downstream primer (10μM), ddH_2O 10μl. The relative quantification of the gene expression was determined using the comparative CT method (2-≥≥Ct).

STATISTICAL ANALYSIS

One-way ANOVA was used to analyze differences between groups. Multiple comparisons were analyzed by Least-Significant Difference (LSD) method. Fisher's exact test was used to analyze qualitative data. A P value that less than 0.05 was regarded as statistically significant. SPSS software was used to analyze all of the data in this article (SPSS 13.0 USA).

RESULTS

Intestine I/R induced mast cell activation and TXL attenuated mast cell activation

Mast cell activation was studied by c-Fos+Trytase immunofluorescence double staining (fig. 1). 3 days after intestine I/R injury, there was an obvious mast cell activation in intestinal mucosa tissue (fig. 6A). TXL low-dose treatment had little difference in mast cell activation compared to I/R group. TXL medium-dose treatment effectively inhibited mast cell activation induced by I/R injury, and showed no difference compared to Sham group. TXL high-dose treatment showed a decreased mast cell activation compared to I/R group but increased compared to Sham group.

7 days after intestine I/R injury, there was an obvious mast cell activation in intestinal mucosa tissue (fig. 6A). TXL low-dose treatment had a little difference in mast cell activation compared to I/R group. TXL medium-dose treatment effectively inhibited mast cell activation induced by I/R injury, and showed no difference compared to Sham group. TXL high-dose treatment showed a similar increased mast cell activation compared to I/R group.

Inflammatory factors and endothelial barrier changes in intestine I/R injury and TXL's inhibitory effect TLR4 mRNA expression changes

RT-PCR analysis of intestine tissue revealed an increased TLR4 mRNA expression in 3 days after intestine I/R injury (fig. 7A). TXL low & medium-dose treatment clearly inhibited over expressed TLR4 mRNA after I/R injury, showed no obvious difference compared to sham group. While, TXL high-dose treatment partly inhibited over expressed TLR4 mRNA after I/R injury.

7 days after intestine I/R injury, there was an obvious increased TLR4 mRNA expression. TXL low-dose treatment could clearly inhibited over expressed TLR4 mRNA after I/R injury and showed no obvious difference compared to sham group. While, TXL medium & high-dose treatment partly inhibited over expressed TLR4 mRNA after I/R injury.

Immunohistochemical analysis for TNF-α

Immunohistochemical staining of intestine tissue revealed an increased TNF-a expression in 3 days after intestine I/R injury (fig. 2; fig. 6B). TXL low, medium & high-dose treatment clearly inhibited over expressed TNF-a after I/R injury. Similar results were seen in 7 days after intestine I/R injury (fig. 2; fig. 6B). Importantly, 7 days intestine I/R injury committed an increased TNF-α expression compared to the 3 days'. It was surprised that 3 doses of TXL treatment exhibited strong inhibition effect on TNF-a over expression after I/R injury.

Immunohistochemical analysis for NF-κB

Immunohistochemical staining was used to analysis NF-κB expression in intestine tissue (fig. 3; fig. 6C). Immunohistochemistry analysis displayed an increased NF-κB expression in 3 days after intestine I/R injury. TXL low, medium & high-dose treatment clearly inhibited over expressed NF-κB after I/R injury. Low-dose treatment showed more effective among 3 TXL treatments. NF-κB expression increased in 7 days after intestine I/R injury compared to sham group, and showed an increase compared with the 3 days'. TXL low, medium & high-dose treatment showed clear and similar inhibition effect over expressed NF-κB after I/R injury.

Immunohistochemical analysis for PECAM-1

Immunohistochemical staining of intestine tissue revealed an increased PECAM-1 expression in 3 days after intestine I/R injury (fig. 4; fig. 6D). TXL low, medium &

Table 1: Composition of Tongxinluo (TXL)

Ingredients (Latin name)	Ingredients (Chinese name)	Family	Part used	Voucher specimen number	Ratio (%)
Insects					
Hirudo nipponica Whitman	Shui zhi	Hirudinidae	Dried body	12,004	27.330
Cryptotympana pustulata Fabricious	Can tui	Cicadidae	Skin	12,005	18.111
Steleophage plancyi (Boleny)	Tu biechong	Corydiidar	Female dried body	12,003	18.111
Buthus martensii Karsch	Quan xie	Buthidae	Dried body	12,002	18.111
Scolopendra subspomopes mutilans L. Koch	Wu gong	Psittacidae	Dried body	12,001	3.623
Plants					
Boswellia carteri Birdw	Ru xiang	Burseraceae	Resin	11,006	5.927
Dalbergia odorifera T. Chen	Jiang xiang	Leguminosae	Heartwood of stem and root	11,005	4.000
Bomeolum syntheticum	Bing pian	Dipterocarpaceae	Resin	11,007	3.626
Panax ginseng C.A.Mey	Ren shen	Araliaceae	Root and rhizome	11,001	1.667
Paeonia lactiflora Pall	Chi shao	Ranunculaceae	Root	11,003	1.558
Ziziphus jujube Mill.Var.spinosa (Bunge)	Suan zaoren	Rhamnaceae	Seed	11,002	1.173
Santalum album L.	Tan xiang	Santalaceae	Heartwood of stem	11,004	0.354

Table 2: Primer Sequence

Primer	Primer sequence
Mus-mir-126-RT Mus-mir-126-F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCATT GCGCTCGTACCGTGAGTAAT
Mus-U6-RT	CGCTTCACGAATTTGCGTGTCAT
Mus-U6-F	GCTTCGGCAGCACATATACTAAAAT
Mus-U6-R	CGCTTCACGAATTTGCGTGTCAT
β-actinF-S	GAGCGTGGCTACAGCTTCACCAC
β-actinF-A	TACTCCTGCTTGCTGATCCACAT
ANGPTL4-F	GCCGCTACTATCCACTAC
ANGPTL4-R	CCTGTTGCTCTGACTGTT
TLR4-F	GCTTTCACCTCTGCCTTCAC
TLR4-R	AGGCGATACAATTCCACCTG

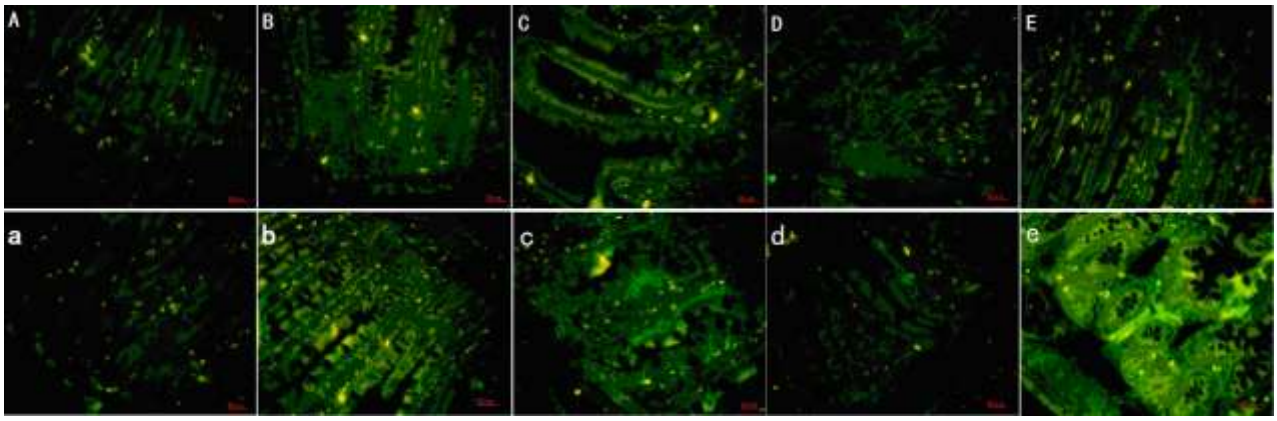

Fig. 1: Intestine Immunofluorescent double staining for c-Fos+Trytase in each group (First row for 3 days Intestine I/R injury; Second row for 7 days Intestine I/R injury. A/a: Sham group; B/b: I/R group; C/c: TXL-L group; D/d: TXL-M group; E/e: TXL-H group; Yellow fluorescence represent for double staining for c-Fos and Trytase, Scale bar = 100 μm).

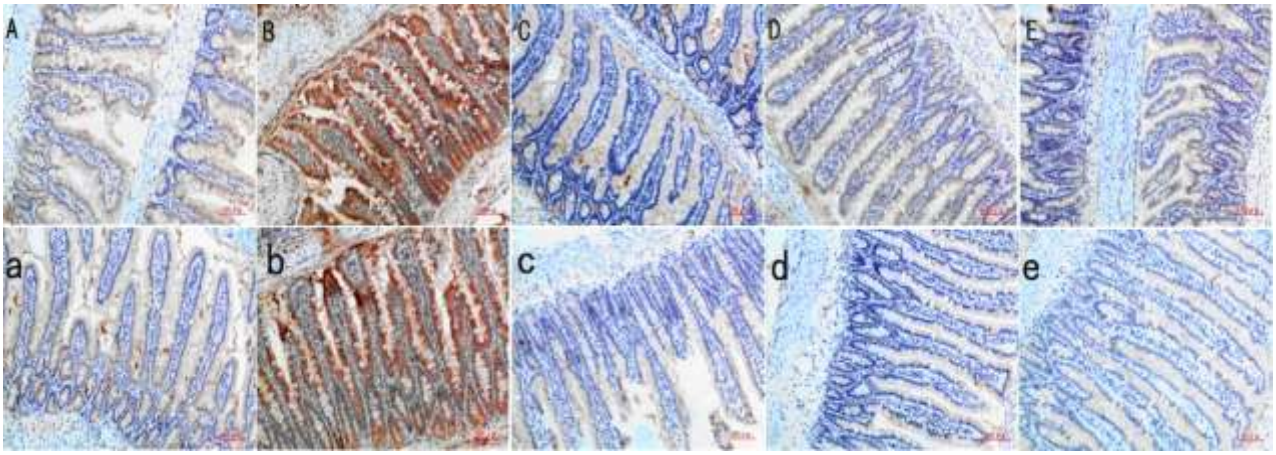

Fig. 2: Intestine Immunohistochemistry staining for TNF-α in each group (First row for 3 days Intestine I/R injury; Second row for 7 days Intestine I/R injury. A/a: Sham group; B/b: I/R group; C/c: TXL-L group; D/d: TXL-M group; E/e: TXL-H group, Scale bar = 100μm).

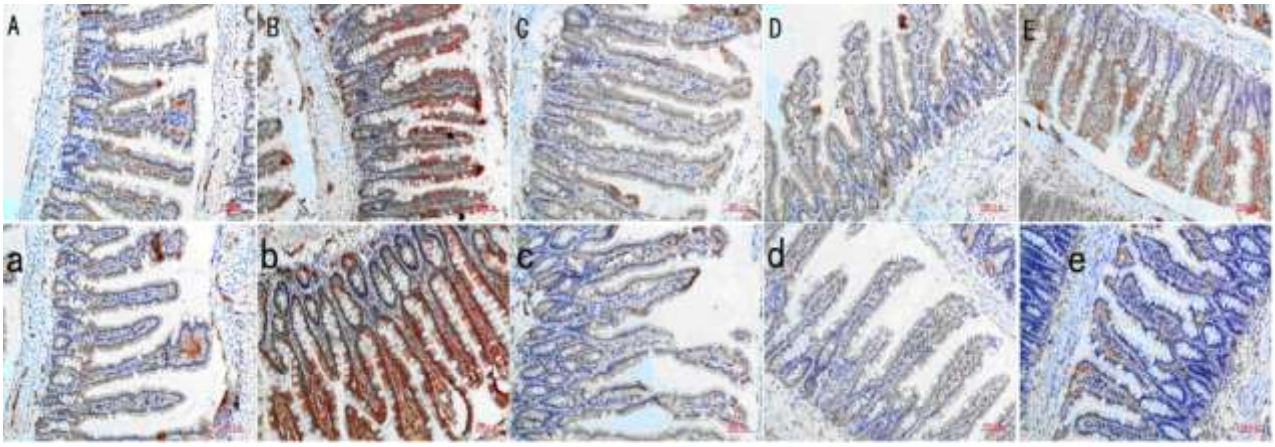

Fig. 3: Intestine Immunohistochemistry staining for NF-κB in each group (First row for 3 days Intestine I/R injury; Second row for 7 days Intestine I/R injury. A/a: Sham group; B/b: I/R group; C/c: TXL-L group; D/d: TXL-M group; E/e: TXL-H group, Scale bar = 100μm).

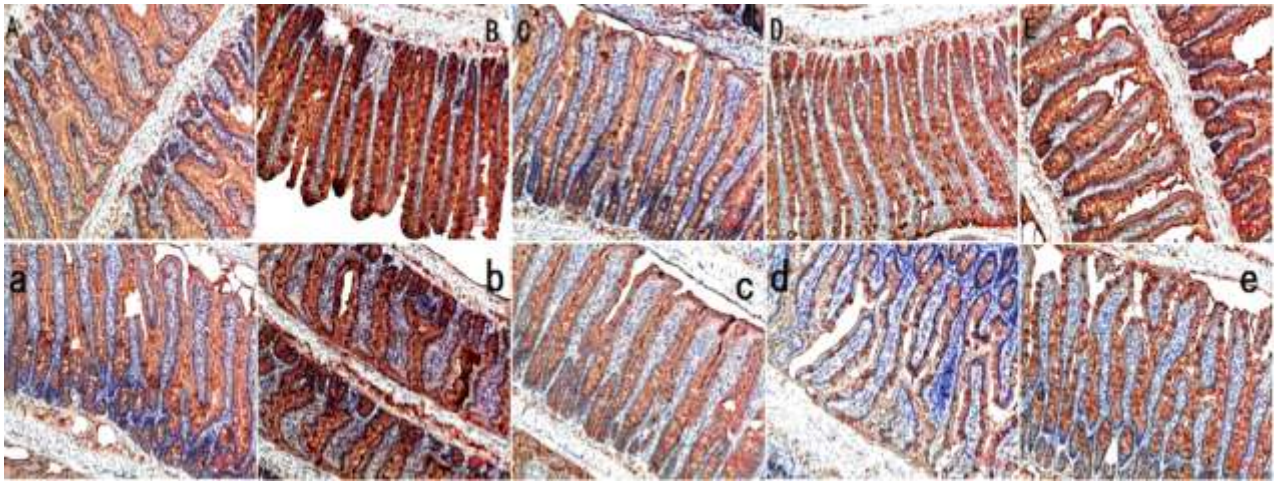

Fig. 4: Intestine Immunohistochemistry staining for PECAM-1 in each group (First row for 3 days Intestine I/R injury; Second row for 7 days Intestine I/R injury. A/a: Sham group; B/b: I/R group; C/c: TXL-L group; D/d: TXL-M group; E/e: TXL-H group, Scale bar = 100 μm).

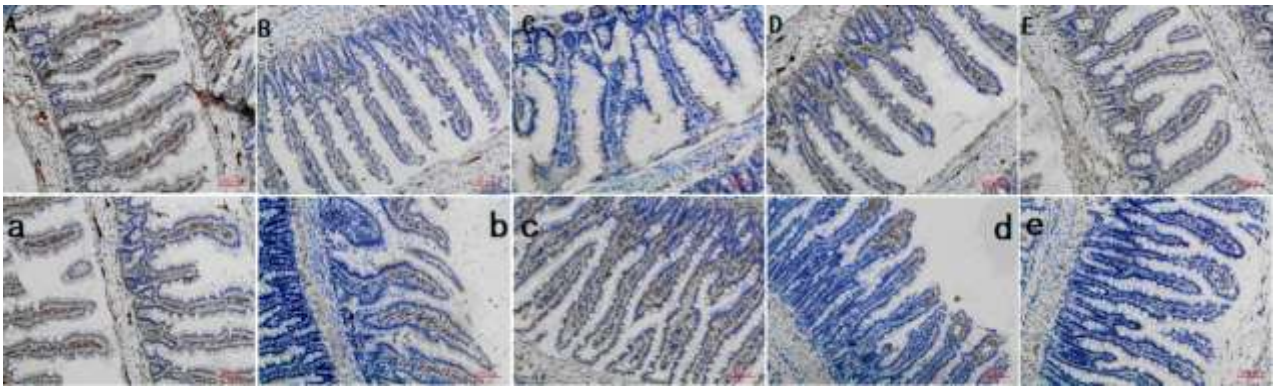

Fig. 5: Intestine Immunohistochemistry staining for VE-Cadherin in each group (First row for 3 days Intestine I/R injury; Second row for 7 days Intestine I/R injury. A/a: Sham group; B/b: I/R group; C/c: TXL-L group; D/d: TXL-M group; E/e: TXL-H group, Scale bar = 100 μm).

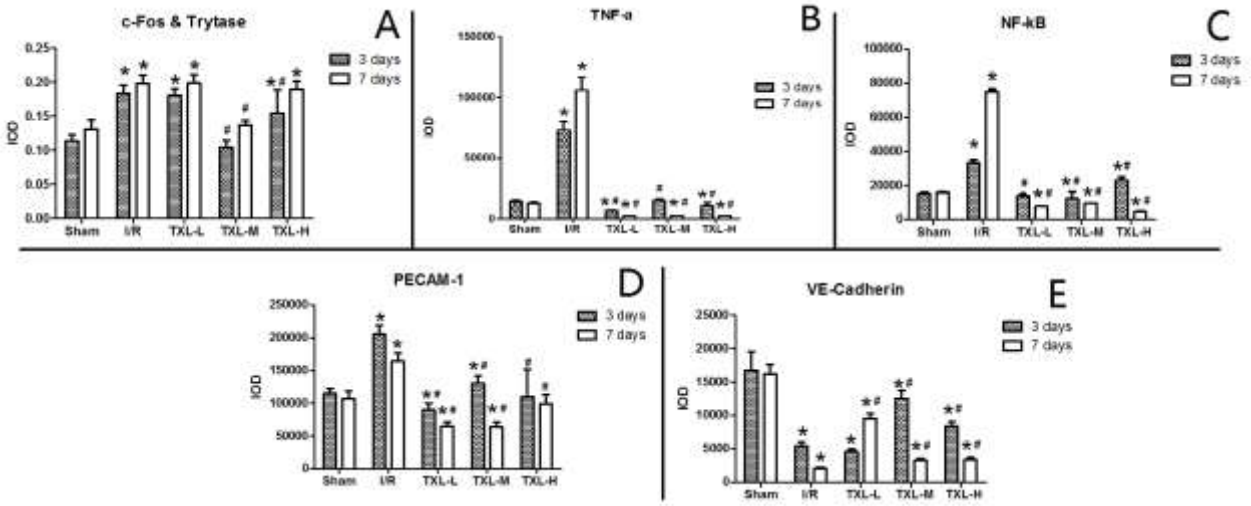

Fig. 6: Quantitative analysis of immunofluorescent double staining (A: c-Fos & Trytase) and immunohistochemical staining (B: TNF-a, C: NF-kB, D: PECAM-1, E: VE-Cadherin) in each group. Data are expressed as means ± SD from 10 rats in each group. *P<0.01 vs. Sham group; #P < 0.01 vs. I/R group.

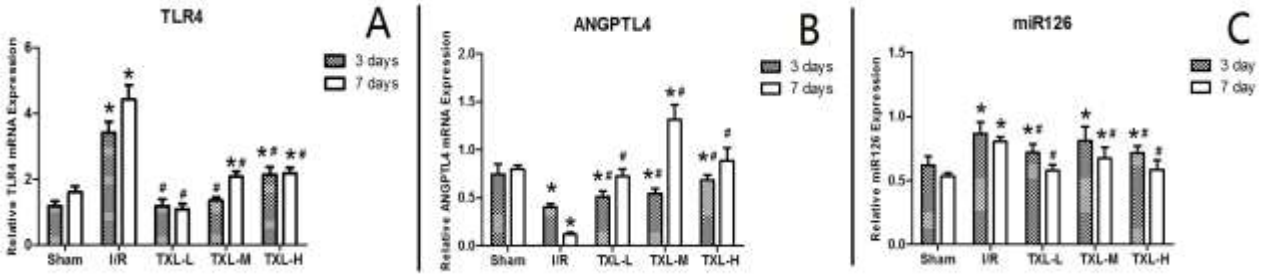

Fig. 7: Quantitative analysis of TLR4 mRNA (A), ANGPTL4 mRNA (B), miR126 (C) expression in each group. Data are expressed as means ± SD from 10 rats in each group. *P<0.01 vs. Sham group; #P < 0.01 vs. I/R group.

high-dose treatment clearly inhibited over expressed PECAM-1 after I/R injury. Low-dose treatment turned out to be most effective. Similar results were showed in 7 days after intestine I/R injury (fig. 4; fig. 6D).

Immunohistochemical analysis for VE-Cadherin

Immunohistochemical staining was used to analysis VE-Cadherin expression in intestine tissue (fig. 5; fig. 6E). Immunohistochemistry analysis displayed a decreased

VE-Cadherin expression in 3 days after intestine I/R injury. TXL low-dose treatment showed no improvement in VE-Cadherin expression after I/R injury. TXL medium & high-dose treatment could partly recover the depressed VE-Cadherin expression induced by I/R injury. TXL medium-dose treatment turned out to be strongest protection against VE-Cadherin lose. Depressed VE-Cadherin expression was also found in 7 days after intestine I/R injury, and worse than the 3 days. In 7 days treatment, TXL low-dose treatment obviously improved depressed VE-Cadherin expression after I/R injury. While, TXL medium & high-dose treatment resulted in mild improvement of VE-Cadherin expression after I/R injury.

Angiogenesis regulatory factors changes in intestine I/R injury and TXL's therapeutic effect ANGPTL4 mRNA expression changes

RT-PCR analysis of intestine tissue revealed a decreased ANGPTL4 mRNA expression in 3 days after intestine I/R injury (fig. 7B). TXL low, medium & high-dose treatment could partly recover the depressed ANGPTL4 mRNA expression after I/R injury. ANGPTL4 mRNA expression in 7 days after intestine I/R injury was further depressed. TXL low, medium & high-dose treatment could fully recover the depressed ANGPTL4 mRNA expression after I/R injury. Besides, medium-dose treatment exerted an over ANGPTL4 expression in 7 days after intestine I/R injury. ANGPTL4 was beneficial for vascular protection. TXL had protective effect on it.

microRNA-126 expression changes

RT-PCR analysis of intestine tissue revealed an increased miR126 expression in 3 days after intestine I/R injury (fig. 7C). TXL low & high-dose treatment could partly inhibited the over expressed miR126 expression after I/R injury. Medium-dose treatment showed no significant changes. miR126 expression in 7 days after intestine I/R injury was similar with 3 days'. However, TXL low, medium & high-dose treatments were more effective than 3 days'. Among them, low & high-dose treatment fully recovered the normal miR126 expression in I/R injured intestine tissue. The results indicated a long-term effect of TXL treatment on I/R induced angiogenesis disorder.

DISCUSSION

Mast cells are widely distributed in the digestive system, mainly around the capillary and lymphatic vessels in the submucosa tissue, accounting for 2% to 3% of the cells in the intestinal mucosa (Bischoff *et al*., 2007). Mast cells are important cells of the innate immune system, studies have confirmed that mast cells is an important part of the inflammatory process, the activation of mast cells release a variety of media, the medium of microenvironment inflammation. Studies have shown that mast cells play an important role in the pathological process of inflammatory bowel disease (Klooker *et al*., 2010). Many studies indicated that mast cell activation plays an important role in intestine I/R induced inflammatory response and tissue damage, and mast cell stabilizers significantly reduced intestine I/R inflammatory response and tissue damage (Boros, 2003). TLRs were mostly expressed in mast cells. By activation of receptors, large amounts of cytokines and chemokines can be released, and the activation of TLR4 pathway induces mast cells releasing a large number of inflammatory factors, including TNF-α, IL-6, etc (Akira *et al*., 2003; Tsan *et al*., 2004). Activated mast cells release TNF-α rapidly (Gordon *et al*., 1990), in the mean time, TLR4 induce the expression of NF-κB via the MyD88 pathway and a large number of NF-κB and TNF-α act as important components of intestine I/R inflammatory response (Wu *et al*., 2009). Immune and inflammation studies confirm that the TLR-NF-κB pathway, including a large number of inflammatory factors such as TNF-α and IL-6, forming a positive feedback loop that further aggravates the inflammatory response in tissues (Campos *et al*., 2004; Aldrich *et al*., 2013). Our results showed that in 3 days intestine I/R injury, mast cells were extensively activated and TLR4, TNF-α, NF-κB expression were significantly increased. Further augments were observed in 7 days intestine I/R injury versus 3 days. Results indicated that mast cell activation and TLR4-NF-κB/TNF-α pathway were widely participated in inflammatory reaction of intestine I/R injury and it persisted in 7 days after intestine I/R injury. TXL treatment significantly reduced the inflammatory response at 3 and 7 days after intestine I/R injury, by inhibiting mast cells activation, decreasing the expression of TLR4 mRNA, TNF-α and NF-κB protein. In general, TXL-medium dose treatment showed the best therapeutic effect.

Inflammatory reaction is closely related to vascular permeability. TNF-α, as a powerful chemotactic factor of granulocyte, promote adhesion molecules adhering to endothelial cells. TNF-α also promote the transfer of granulocytes through the vascular barrier to the inflammation site (Sun *et al*., 2000). PECAM-1 is expressed in platelets, leukocytes and endothelial cells, involved in the process of leukocyte exudation. PECAM-1 is closely related to the permeability of endothelial cells. Studies have shown that anti-PECAM-1-MAb can reduce leukocyte recruitment and endothelial permeability injury after intestine I/R injury (Sun *et al*., 2000; Franciose *et al*., 1996). VE-cadherin, a tight junction protein of endothelial cells, is critical for the maintenance of endothelial barrier structure and function. Inhibition of VE-cadherin expression or its adhesion function experiments have confirmed that VE-cadherin is an important adhesion molecule to form tight junctions and maintain endothelial barrier (Gavard *et al*., 2006; Heupel *et al*., 2009; Hebda *et al*., 2013). Our results showed an increased PECAM-1 protein expression and decreased VE-cadherin protein expression in both 3 and 7 days intestine I/R injury.

Especially, VE-cadherin expression was further decreased in 7 days injury versus 3 days', indicated severe vascular permeability and endothelial barrier damage of intestine I/R injury within 7 days. TXL treatment inhibited PECAM-1 over expression and recovered VE-cadherin expression to protect vascular structure and function in favor of attenuation of intestine I/R induced inflammation. TXL-low and medium dose treatment had the better therapeutic effect.

ANGPTL4 belongs to a multifunctional protein of the angiopoietin like protein family. Ischemic and hypoxia stimulates ANGPTL4 expression (Murata *et al*., 2009). Studies have shown that ANGPTL4 promote angiogenesis in a variety of pathological conditions (Hermann *et al*., 2005; Perdiguero *et al*., 2011). ANGPTL4 produced by hypoxia tissue can improve endothelial barrier function (Cazes *et al*., 2006). MiR126 is one of the mostly expressed microRNA in endothelial cells that have been identified (Voellenkle *et al*., 2012). MiR126 has the function of protecting endothelial cells integrity and promoting angiogenesis in injury tissue (Fish *et al*., 2008). Our results showed a decrease of ANGPTL4 expression and increase of MiR126 expression in 3 and 7 days intestine I/R injury. TXL treatment could recover ANGPTL4 expression intestine I/R injury and had no obvious treatment effect on MiR126, which indicated TXL's positive effect on vascular regulation in intestine I/R injury.

CONCLUSION

Mast cell activation was observed in intestine I/R injury within 7 days, along with TLR4-NF-κB/TNF-α signal pathway activation. Those changes contributed to inflammation in intestine I/R injury. Besides, endothelial barrier dysfunction and enhanced vascular permeability were detected in intestine I/R injury within 7 days. TXL treatment attenuated mast cell activation and overexpression of related TLR4-NF-κB/TNF-α signal pathway, which contributed to attenuate inflammation in intestine I/R injury. TXL treatment alleviated vascular permeability and protected endothelial barrier function against intestine I/R injury, which were helpful to reduce inflammation in intestine I/R injury. In addition, TXL could enhance ANGPTL4 gene expression, which may benefit endothelial newborn and sustain.

Abbreviations

TXL: Tongxinluo; I/R: Ischemia-reperfusion; TCM: Traditional Chinese medicine; TLR4: Toll-like receptor 4; IL-1: Interleukin-1; IL-6: Interleukin-6; IL-12: Interleukin-12; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; SPF: Specific pathogen free; PLAGH: People's Liberation Army General Hospital; PECAM-1: Platelet endothelial cell adhesion molecule-1; VE-Cadherin: Vascular endothelial-cadherin; IOD: Integral optical density; ANGPTL-1: Angiopoietin-1; LSD: Least-Significant Difference.

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