

Effect and mechanism of omega-3 polyunsaturated fatty acids on intestinal injury in rats with obstructive jaundice

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Abstract. – **OBJECTIVE:** Obstructive jaundice (OJ) is a common clinical pathological syndrome in hepatobiliary surgery. High incidence of multiple organ injuries during perioperative period and its associated mortality remains challenging in clinical practice. Omega-3 polyunsaturated fatty acids (ω -3 PUFA) is an important enteral immune nutrition. This study investigated the protective role of ω -3 PUFA in the regulation of inflammatory response in OJ.

MATERIALS AND METHODS: Seventy-two rats were randomly divided into obstructive jaundice (OJ) group, obstructive jaundice + ω -3 PUFA group (OJPUFA) group, and sham group. OJ model was created by ligation of the bile duct. Abdominal thoracic catheter was placed to collect lymph. Body weight, liver function, serum and lymphatic levels of TNF- α , IL-1 β , IL-10, HMGB1, and nitric oxide (NO) were measured on day 3, day 7, and day 14 after operation. Hematoxylin staining and Alcian blue-periodic acid-Shiff (AB-PAS) staining were performed on the ileum tissue. Protein and mRNA expression of HMGB1, TLR4, and NF- κ B p65 were measured at the aforementioned time points.

RESULTS: The general condition, including body weight and liver function, were worse in the OJ and the OJPUFA group compared to that in the sham group. On day 14, the body weight recovery and liver function were significantly better in the OJPUFA group than those in the OJ group were ($p < 0.05$ for all). No marked change in the serum and lymphatic levels of TNF- α , IL-1 β , IL-10, HMGB1 and NO was observed in the sham group after operation, while corresponding levels in the OJ and the OJPUFA groups were significantly higher. Compared with the OJPUFA group, serum and lymphatic levels of the above factors were consistently higher in the OJ group and were significantly higher on day 14 ($p < 0.05$ for all). At the same time, ω -3 PUFA lowered the damage of intestinal villi and intestinal muco-

sal epithelium. It also improved the number and function of goblet cells in intestinal mucosal epithelium. The protein and mRNA expression of HMGB1, TLR4, and NF- κ B p65 were significantly higher in the OJ group than those in the OJPUFA group ($p < 0.05$ for all).

CONCLUSIONS: ω -3 PUFA has protective effect in the management of obstructive jaundice. It can regulate the inflammatory response and reduce its damage to intestinal structure. Reducing the activation of HMGB1/TLR4/ NF- κ B pathway might be a mechanism for its protective effect. We suggested that ω -3 PUFA and drugs targeted HMGB1/TLR4/NF- κ B pathway might be potential treatment strategies in obstructive jaundice.

Key Words:

Inflammatory response, Multiple organ dysfunction, Obstructive jaundice, ω -3 PUFA.

Introduction

Obstructive jaundice (OJ) is a common clinical pathological syndrome in hepatobiliary surgery. In recent years, basic studies have found that damage to intestinal mucosal barrier function and liver reticular endothelial cell dysfunction are two key components in bacterial/endotoxin migration^{1,2}, and may later contribute to multiple organ dysfunction after OJ^{3,4}. In OJ patients, their mechanical, immune, biological, and chemical barrier that constitute the intestinal barrier function are damaged to varying degrees. The damage results in increased intestinal mucosal permeability and increased endotoxin absorption⁵⁻⁸. Meanwhile, the intestinal endotoxin clearance capacity is impaired⁹. In addition, the gut microbiome dy-

namic balance is affected, which also facilitates bacterial translocation¹⁰. Eventually, gut-derived endotoxemia induces multiple organ damage¹¹.

At present, research on the initiation factors of systemic damage of the OJ mainly focus on the intestinal mucosal barrier dysfunction and the translocation of bacteria/endotoxin. Recent studies^{12,13} have confirmed that after intestinal mucosal barrier dysfunction, lipopolysaccharide (LPS) and other inflammatory factors have direct contact with intestinal epithelial cells. The contact might activate intestinal epithelium and intestinal related lymphoid tissues and lead to intestinal cytokines release^{14,15}. These inflammatory cytokines may enter the systemic circulation via the lymph pathway and result in systemic damage^{16,17}. The lymphatic system plays an important role in maintaining the stability of the body environment. In recent years, the “intestinal lymphatic” pathway has been discovered to participate in the systemic inflammatory response in critical conditions, such as hemorrhagic shock, ischemia-reperfusion injury, severe burns, sepsis and acute pancreatitis¹⁸⁻²⁰. In the aforementioned pathological process, cytokines and inflammatory mediators in the intestine enter the systemic circulation, participate in the systemic inflammatory response and affect multiple tissues and organs. However, the role of intestinal lymph circulation in the pathophysiology of OJ is less studied. One reason might be the difficulty in obtaining lymph. At present, simple and mature lymph collection techniques are still lacking.

Omega-3 polyunsaturated fatty acids (ω -3 PUFA) is an important immune enteral nutrition. *In vitro* and *in vivo* studies²¹⁻²⁴ have confirmed that ω -3 PUFA can inhibit immunity cell proliferation and activation and reduce pro-inflammatory cytokine production. It has immunomodulatory effects by inhibiting expression of adhesion molecules and affecting other pathways. Effects of omega-3 PUFA are further supported by some clinical studies^{25,26}. It is possible that ω -3 PUFA might regulate the inflammatory response in patients with OJ and affect the expression of high-mobility group protein 1 (HMGB1) and other inflammatory factors. However, no study so far has checked the association.

First of all, we aimed to establish OJ rat model and insert thoracic duct catheter to collect lymph in this project. Second, after successfully performed the first step, we assessed the effect of ω -3 PUFA rich fish oil on serum and lymphatic levels of cytokines including tumor necrosis fac-

tor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-10 (IL-10), nitric oxide (NO) and HMGB1. Third, we further measured the expression of aforementioned cytokines and inflammatory factors in the intestine. Fourth, we analyzed change in expression of HMGB1 and Toll-like receptor-4 (TLR4) and nuclear factor- κ B (NF- κ B) protein in intestinal mucosa tissue. Overall, we aimed to explore the role of “intestinal-lymph pathway” and HMGB1 in the intestinal barrier dysfunction caused by OJ and explore the mechanism of the potentially protective effect of ω -3 PUFA in OJ animal models.

Materials and Methods

Animals

All animal experiments in this study complied with the guidelines for animal experiment of the Ethics Committee of Binzhou Medical College. All specific pathogen-free (SPF) male Wistar rats (Shandong Lukang Pharmaceutical Co., Ltd. Experimental Animal Center, Shandong, China) were housed in standard animal maintenance cages at the Experimental Animal Center of Binzhou Medical College. All animals were housed at 22-26°C with relative humidity 40%-70% and 12h light/dark cycle. Each cage had 3 rats. Rats had full access to food and water. After 14 days of adaptive feeding, 72 rats with weight 200-240 grams were randomly divided into the following 3 groups using random number table: obstructive jaundice (OJ) group (n = 24), obstructive jaundice + ω -3 PUFA group (OJPUFA) group (n = 24), and sham group (n = 24).

Surgical Operation

The rats were fasted for 6 hours but with full access to water before the operation. They were anesthetized by intraperitoneal injection with sodium pentobarbital diluted in saline (40 mg/kg). After anesthesia, a surgical incision of approximately 2 cm from the midline of the xiphoid abdomen was taken and sequentially cut into the abdomen. The hepatoduodenal ligament was then revealed. After carefully exposing the portal vein, common bile duct and bile duct to the hilar, obstructive jaundice was induced by double ligation of the bile duct at the distal end. In the sham group, the bile duct was similarly exposed, but no ligation was performed. All animals had access to water at 6h after operation and were fed 24h after operation. Each rat was housed in its own cage

and under the same standard conditions as mentioned previously. Animals in the OJPUFA group received fish oil at a dose of 0.4 g/kg*d starting from the first day after surgery. The dosage of fish oil was determined based on previous studies²⁷⁻³⁰. No other drugs were administered with fish oil. Animals in the other groups were given the same amount of saline at the corresponding time points. Mental and activity status, eating, defecation (urine and stool), jaundice, and changes in body weight were recorded.

Collection of Lymph

Eight rats in each group were sacrificed on day 3, day 7, and day 14 after operation. Lymph, inferior vena cava blood, and ileal tissue were collected. One rat in the OJ-14d group died of intestinal obstruction before planned sacrifice.

Rats were anesthetized as described before. After satisfactory anesthesia, a midline incision was taken and extended to about 4 cm. Abdominal

cavity was carefully observed for changes in ascites, liver, and intestines. Left kidney was exposed and the posterior peritoneum was cut at the level of the upper left pole of the kidney to separate and expose the abdominal aorta. In the left rear of the abdominal aorta or the left parallel to the abdominal aorta, or even the right posterior position of the abdominal aorta in some rats (Figure 1A), thoracic duct was exposed. Lymph was observed as pale milky white (the sham group) or yellow (the OJ or the OJPUFA group) fluid. We then separated the chest catheter carefully along the abdominal aorta to avoid clamping or pulling the chest catheter. The lymphatic vessels were carefully separated at the level of the left renal vein and the diaphragmatic foot. Thoracic duct was then ligated close to the diaphragmatic foot. A 4Fr or 5Fr peripherally inserted central catheter (PICC) catheter was trimmed to about 6 cm and to a 45° wedge on one side and then rinsed with heparin. Thoracic duct was cut near the first suture posi-

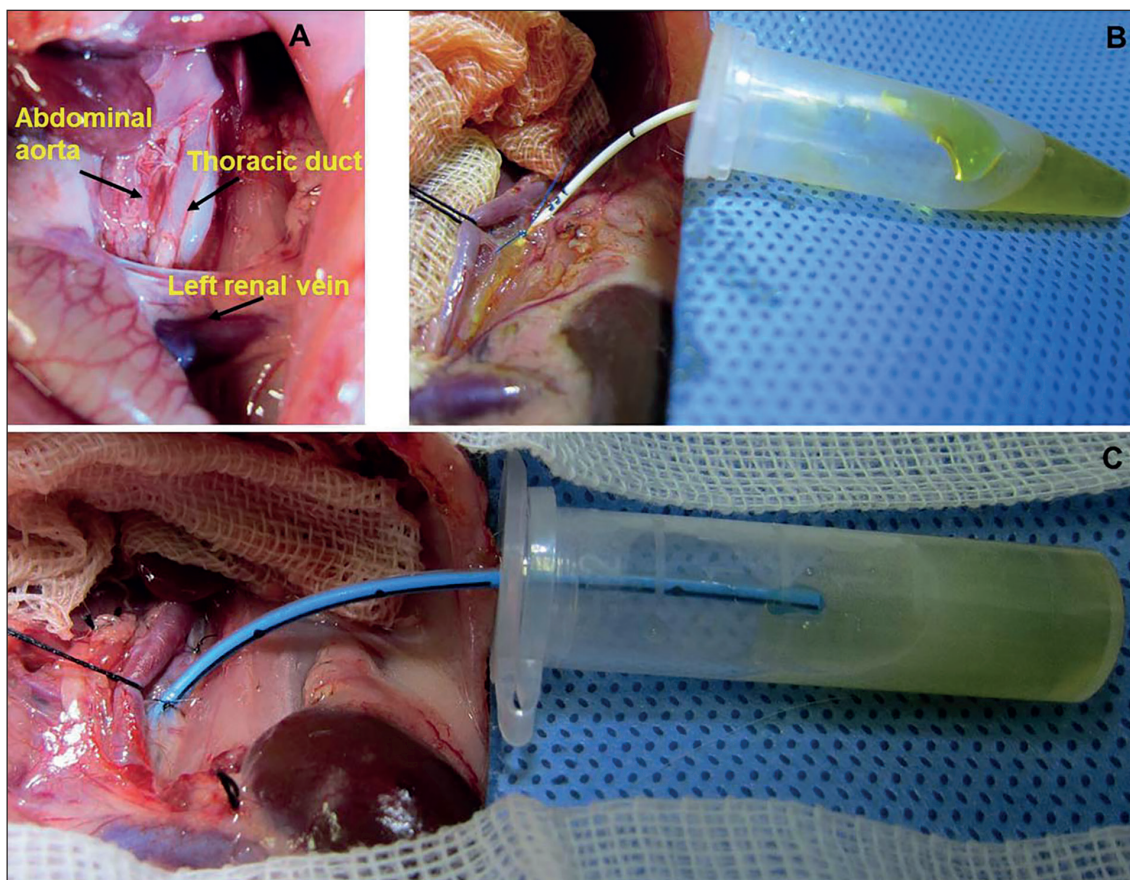


Figure 1. Surgical operation. Anatomy position of abdominal aorta, thoracic duct, and left renal vein (A). After the catheter was successfully inserted, lymph was collected in an EP tube with yellow color in the OJ group (B), similar in the OJPUFA group) or milky white color in the sham group (C).

tion at the distal end and the PICC catheter was placed into about 2-4 mm. The lymphatic tube was then sutured to the left vertebra. The catheter was drawn to the outside. Abdominal wall was temporarily closed, and the surgical incision was moistened with saline gauze. The outside end of the catheter was inserted into an EP tube. Lymph was collected for 45 minutes (Figure 1B for the OJ group, and Figure 1C for the sham group). After the collection, catheter was removed. The supernatant of the lymph was stored in -80°C after centrifuge.

Blood and Bowel Tissue Collection

About 2-3ml of blood was collected from the inferior vena cava and then centrifuged. The supernatant was stored in -80°C. After the blood collection was completed, about 3 cm away from the ileocecal area, 3 sections of ileum to the proximal end were collected. The tissue was cleaned with saline. About 1 cm of ileum closest to the proximal end was placed in 1mL TRIzol. The second 1 cm ileum was quick frozen by liquid nitrogen. The third 1 cm of ileum was fixed with 10% neutral formaldehyde and embedded with paraffin. All ileum tissue samples were stored in -80°C.

Laboratory Test

Liver function indicators, including total bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured by automatic biochemical detector (Beckman Coulter, Brea, CA, USA). Levels of TNF- α , IL-1 β , IL-10 and HMGB1 were measured by Enzyme-Linked Immunosorbent Assay (ELISA, Uscn Life Science Inc, Wuhan, Hubei, China) according to the commercial kit's instructions. Nitrate reductase (microplate method, Nanjing Jiancheng Biotechnology Research Institute, Nanjing, Jiangsu, China) was used to measure the level of NO according to the instruction of the commercial kit.

Histology

Hematoxylin staining and Alcian blue-periodic acid-Shiff (AB-PAS) was performed on the paraffin-embedded ileum tissue to characterize the intestinal mucosa morphology and change in goblet cell. Goblet cell density was then measured by absorbance value with wavelength of A570 nm. Immunohistochemical staining was used to measure the change in expression of HMGB1, TLR4

and NF- κ b p65 in the intestinal mucosa per instruction of the commercial kit (Zhongshan Jinqiao Life Science Inc, Wuhan, Hubei, China).

Quantitative Reverse Transcription PCR (qRT-PCR)

qRT-PCR was used to measure the mRNA expression of HMGB1, TLR4, and NF- κ b p65 in the ileal tissue. RNA was extracted using Total RNA Extractor kit (Sangon biotech, Shanghai, China). cDNA synthesis was performed as instructed by commercial cDNA synthesis kit (Sangon biotech, Shanghai, China). The ABI 7500 Fast real-time PCR system (ABI, USA) was used for qRT-PCR.

An endogenous internal control (GAPDH) was applied to calculate relative expression. The primer sequences were: HMGB1 forward primer, 5'-TGGCGGCTGTTTGTGTCATT-3'; HMGB1 reverse primer, 5'-TGTGACCCAAAATGGGCAAAAGC-3'; TLR4 forward primer, 5'-TTCTGAGTAGCCGCTCTGGC-3'; TLR4 reverse primer, 5'-TGCCTCCCCAGAGCATTGTC-3'; NF- κ B forward primer, 5'-AAGTACCCGGATACAGCAGC-3'; NF- κ B reverse primer, 5'-CAGACGCTGGCCTCCAAACA-3'; GAPDH forward primer, 5'-CCAGGGCTGCCTTCTCTTGT-3'; GAPDH reverse primer, 5'-GTGCCGTTGAACTTGCCGTG-3'.

Western-Blot

Western-blot was performed to measure the protein expression of HMGB1, TLR4, and NF- κ b p65 in the ileal tissue. Briefly, the bicinchoninic acid assay (BCA) assay (BiYunTianSheng Bio-Tech, Shanghai, China) was used to measure protein concentration. Equal amount of protein (100 ug per lane) was first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membrane was blocked with 5% non-fat milk and incubated with primary antibody in Tris Buffered Saline with Tween (TBST) at 4°C overnight. The primary antibodies were comprised of rabbit anti-HMGB1 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti- NF- κ B p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. After incubation, blots were incubated with anti-rabbit IgG HRP-conjugated antibodies with 1:3000 dilution for 60 min at room temperature. The intensity of bands was measured with a scanning densitometer coupled with Image-Pro Plus.

Statistical Analysis

SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Continuous variables were expressed as mean (standard deviation, SD) if following normal distribution, and median (interquartile range, IQR) if not. Shapiro-Wilk test was performed to assess the normality. Between-group comparison was performed by Student *t*-test or one-way ANOVA. Least significant difference (LSD) was used for post-hoc comparison if homoscedasticity assumption was met, and Dunnett's T3 comparison was used if not. A two-sided $p < 0.05$ was treated as statistically significant.

Results

General Condition

No complications such as postoperative incision infection or incision dehiscence occurred in any animals. One rat in the OJ-14d group died of intestinal obstruction before planned sacrifice. In terms of lymphatic catheterization, the procedure failed only in one case in the Sham-d3 group due to intraoperative damage of the abdominal aorta. The procedure was successfully performed in the rest of rats.

After bile duct ligation, the rats had limited movement and loss of appetite. Especially in the OJ group and the OJPUFA group, the weight of rats decreased on day 3 after the operation but returned to the preoperative level on day 7. At any

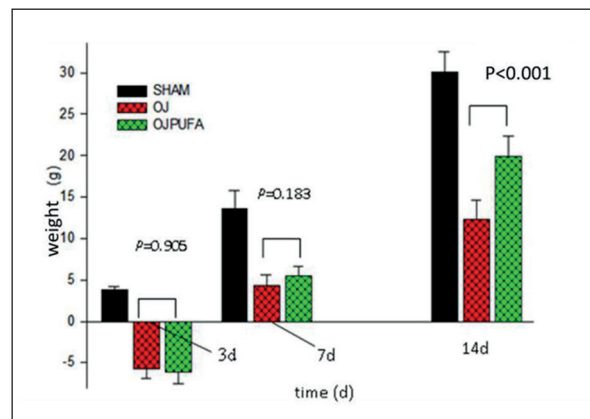


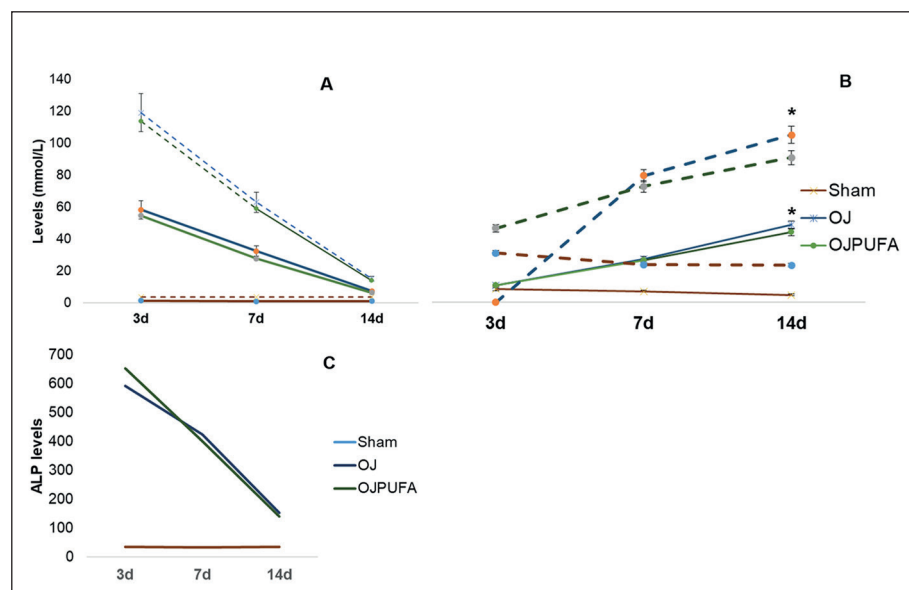
Figure 2. Body weight of rats in each group on day 3, 7 and 14 after surgical operation.

same time point after operation, the weight of rats in the OJ group and the OJPUFA group was significantly lower than that in the sham group ($p < 0.001$, Figure 2). On day 3 and day 7 after operation, there was no significant difference in body weight between the OJ group and the OJPUFA group. On day 14, the body weight of the rats in the OJPUFA group was higher than that in the OJ group ($p < 0.001$).

Change in Liver Function

The levels of serum total bilirubin (TBIL, Figure 3A, dashed line) and direct bilirubin (DBIL, Figure 3B, solid line) in the sham group did not change significantly throughout the experiment.

Figure 3. Change in serum total bilirubin (A, solid line), direct bilirubin (A, dashed line), alanine aminotransferase (ALT, B, solid line), aspartate aminotransferase (AST, B, dashed line), and serum ALP level, C) after operation. * $p < 0.05$ for comparison between the OJ and the OJPUFA group.



Serum TBIL and DBIL levels in the OJ group and the OJPUFA group were significantly higher than those in the sham group on day 3, day 7 and day 14 ($p < 0.001$ for all). At any time, serum TBIL and DBIL levels in the OJ group were slightly higher than those in the OJPUFA group were, but the differences were not significant ($p > 0.05$ for all).

The levels of ALT (Figure 3A, solid line) and AST (Figure 3B, dashed line) in the sham group gradually decreased after operation. Serum ALT and AST levels in the OJ group and the OJPUFA group were higher than those in the sham group on day 3 after surgery, but there was no significant difference ($p > 0.05$ for all). Over time, the serum levels of ALT and AST in the OJ group and the OJPUFA group gradually increased. On day 14, ALT and AST levels in the OJ group were significantly higher than those in the OJPUFA group ($p = 0.014$ for ALT and $p = 0.012$ for AST). Similar trend of change was observed in serum ALP levels (Figure 3C).

Change in Serum and Lymphatic Levels of Inflammatory Factors

In the sham group, the levels of TNF- α in the serum (Figure 4A, solid line) and lymph (Figure 4B, dashed line) gradually decreased after operation and were lowest on day 14. In contrary to the sham group, serum and lymphatic TNF- α levels in the OJ and the OJPUFA groups reached the highest value on day 7 and then decreased. At each time point, serum and lymphatic TNF- α

levels were significantly higher than those in the sham group ($p < 0.05$ for all).

On day 3 and day 7, serum TNF- α levels in the OJPUFA group were lower than those in the OJ group, but the difference was not statistically significant. However, on day 14, serum TNF- α level in the OJPUFA group was significantly lower than that in the OJ group ($p = 0.014$). Change in lymphatic levels of TNF- α was consistent with change in serum levels of TNF- α . On day 14, TNF- α level in lymph fluid in the OJPUFA group was significantly lower than that in the OJ group ($p = 0.035$).

Similar trends of change were observed in serum and lymphatic IL-1 β (Figure 4B, solid line for serum level and dashed line for lymphatic level) and serum and lymphatic IL-10 levels (Figure 4C). Serum and lymphatic levels of IL-1 β and IL-10 in the OJ and the OJPUFA group were significantly higher than those in the sham group ($p < 0.05$ for all). On day 14, serum and lymphatic levels of IL-1 β and IL-10 in the OJPUFA group were significantly lower than those in the OJ group ($p < 0.05$ for all).

Change in Serum and Lymph Levels of HMGB1 and NO

After operation, serum and lymphatic levels of HMGB1 (Figure 5A) and NO (Figure 5B) in the sham group decreased gradually. Consistently at each time point, serum and lymphatic levels of HMGB1 and NO were significantly higher in the OJ and the OJPUFA group than those in the sham group ($p < 0.05$

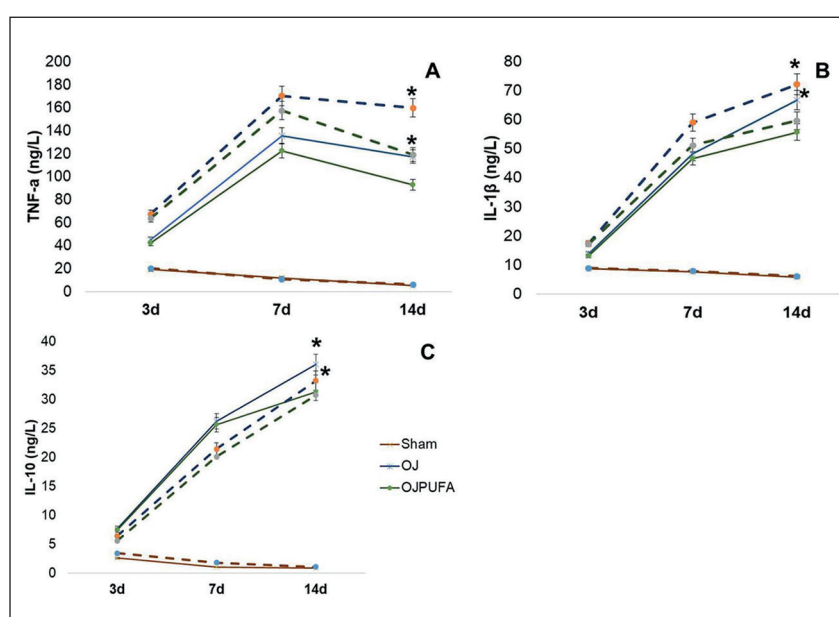


Figure 4. Change in TNF- α (A), IL-1 β (B), and IL-10 (C) after operation. Solid lines for serum levels, and dashed lines for lymphatic levels, respectively.

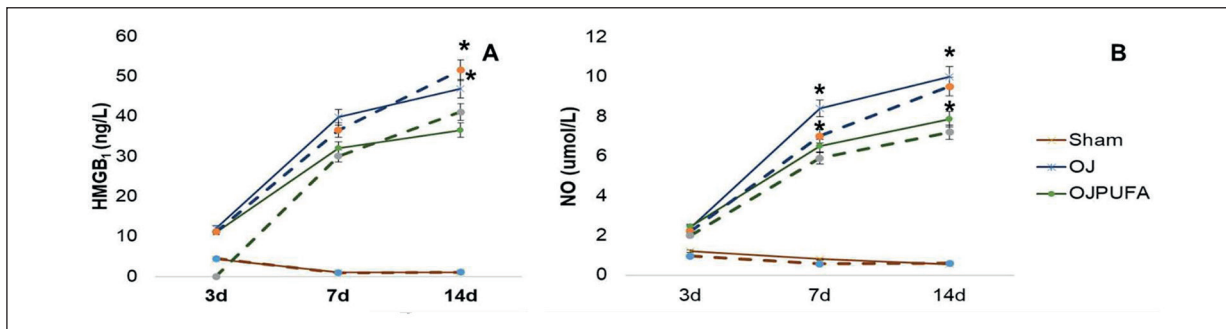


Figure 5. Change in HMGB1 (A) and NO (B) after operation. Solid lines for serum levels, and dashed lines for lymphatic levels, respectively.

for all). On day 14, serum and lymphatic levels of HMGB1 were significantly higher in the OJ group than those in the OJPUFA group ($p < 0.05$ for both). While on day 7 and day 14, serum and lymphatic NO levels were significantly higher in the OJ group than those in the OJPUFA group ($p < 0.05$ for all).

Intestinal Histopathological Changes

In the sham group, the structure of intestinal villi was intact and with a smooth surface (Figure 6A and 6B). No congestion, edema, or inflammatory cell infiltration was observed in the stroma. In the OJ group, villi edema, stroma congestion, and in-

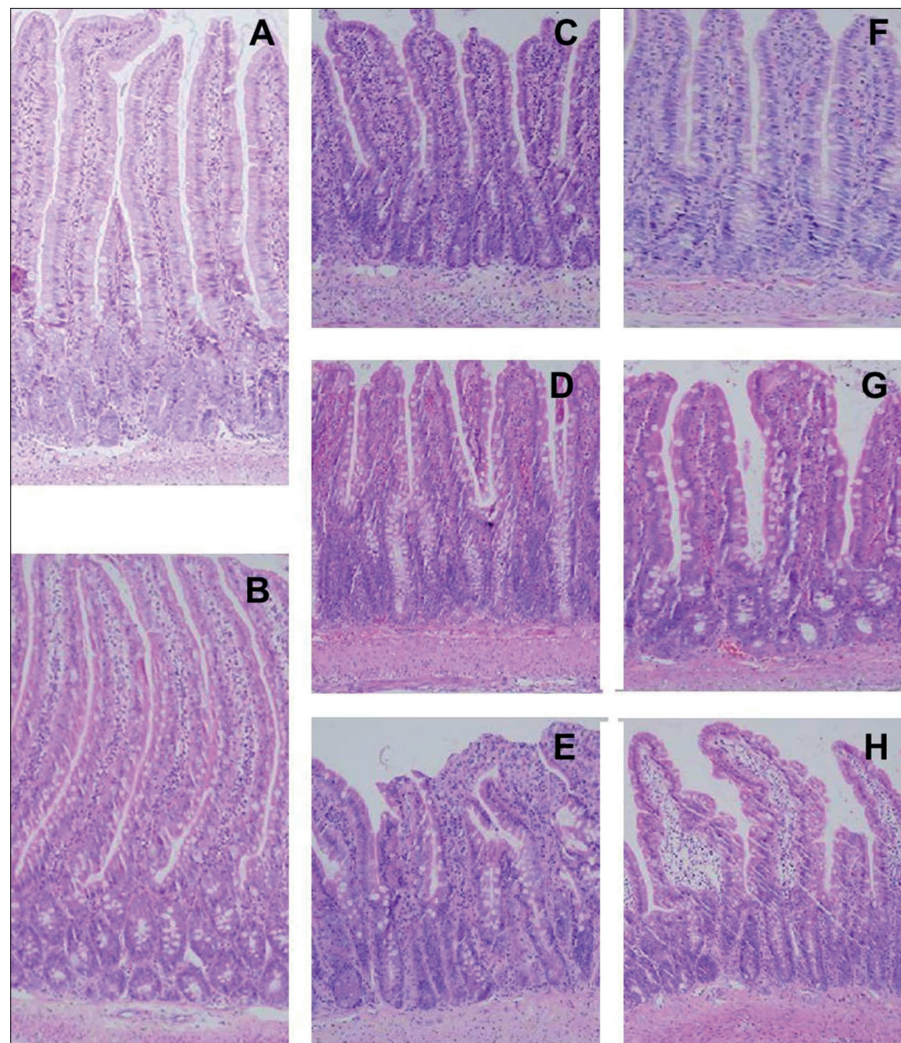


Figure 6. HE staining of intestinal tissue (*200 magnification). The sham group on day 3 (A), the sham group on day 14 (B); the OJ group on day 3 (C), the OJ group on day 7 (D), the OJ group on day 14 (E), the OJPUFA group on day 3 (F), the OJPUFA group on day 7 (G), and the OJPUFA group on day 14 (H).

Table I. Villus length, crypt depth, and ratio of villus length/crypt depth in the three groups.

	Villus length (μm)	Crypt depth (μm)	Ratio of villus length/crypt depth
Sham	463.3 ± 15.1	136.0 ± 4.9	3.41 ± 0.10
OJ	291.6 ± 20.5	105.6 ± 9.1	2.7 ± 0.33
OJPUFA	369.7 ± 28.2	122.7 ± 9.8	3.03 ± 0.35

inflammatory cell infiltration gradually deteriorated after operation. At the same time, villus shrinkage was observed with a decreasing ratio of the villus length to the crypt depth (Figure 6C and 6D). On day 14, marked villus shrinkage was observed in the OJ group (Figure 6E). At the same time, in the OJ group, part of the villi epidermis was missing, and ulcers were formed in some areas. In the OJPUFA group, congestion and edema were significant on day 3 and day 7 (Figure 6F and 6G). On day 14, villus shrinkage was clear, but the structure remained relatively intact, with minimal villous shedding or ulcer formation (Figure 6H).

Villus length in the sham group was significantly longer than those in the OJ and the OJPUFA group on day 14 ($p < 0.001$, Table I). Villus length in the OJPUFA group was significantly longer than that in the OJ group ($p < 0.001$). The trend of change of crypt depth was similar to the change of villus length. However, the difference of the ratio in the OJPUFA group and the OJ group did not reach statistical significance ($p = 0.11$).

In the sham group, goblet cells were uniformly distributed in the intestinal mucosal epithelium (Figure 7A, B, and C). In OJ and the OJPUFA groups, villous edema was significant and the

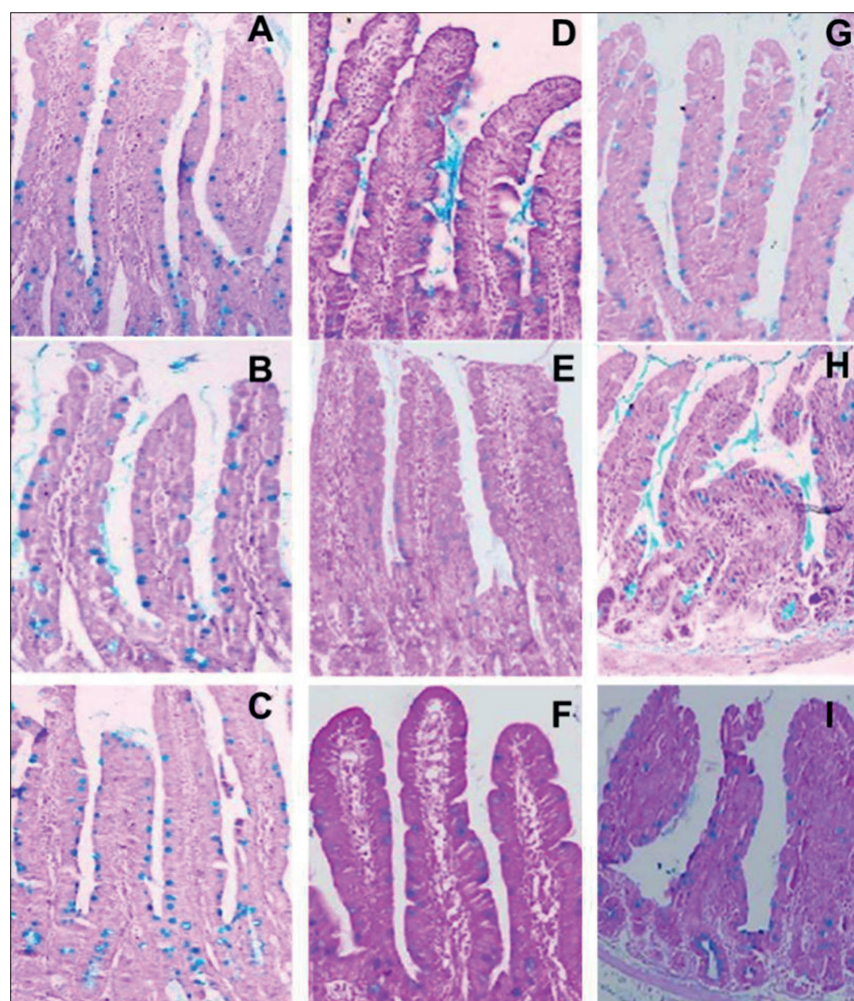


Figure 7. AB-PAS staining of goblet cells intestinal tissues (*200 magnification). The sham group on day 3 (A), the sham group on day 7 (B); the sham group on day 14 (C); the OJ group on day 3 (D), the OJ group on day 7 (E), the OJ group on day 14 (F), the OJPUFA group on day 3 (G), the OJPUFA group on day 7 (H), and the OJPUFA group on day 14 (I).

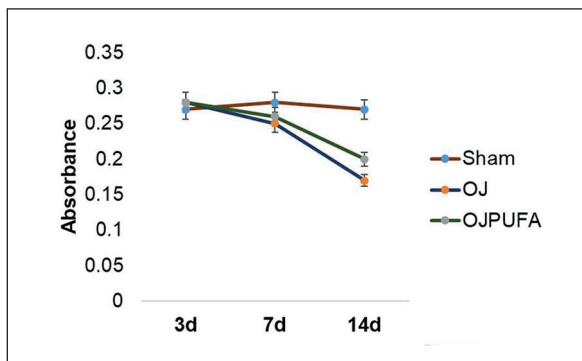


Figure 8. Absorbance value (A570 nm) measuring density of goblet cells.

absorbance values of goblet cells on day 3 was slightly higher than that in the sham group (Figure 7D and 7G). Significant decrease in the number of goblet cells was observed on day 7 (Figure

7E, 7H and Figure 8) and day 14 (Figure 7F and 7I) in the OJ and the OJPUFA group. A more dramatic decrease in the number of goblet cells was observed in the OJ group compared to that in the OJPUFA group on day 14 ($p=0.035$).

Expression of HMGB1

In the sham group, relative low expression of HMGB1 was found in intestinal epithelial cells post operation (Figure 9A, 9B, and 9C). In the OJ and the OJPUFA group, the staining was evident starting on day 3 (Figure 9D and 9G) and was mainly at the apical villi and in the nucleus of intestinal epithelial cells. Interstitial inflammatory cells also showed HMGB1 expression. On day 7, in the OJ group, HMGB1 expression was significant in the nucleus and cytoplasm of the entire villous epithelial cells and in the stroma of infiltrated inflammatory cells (Figure 9E). The expression in

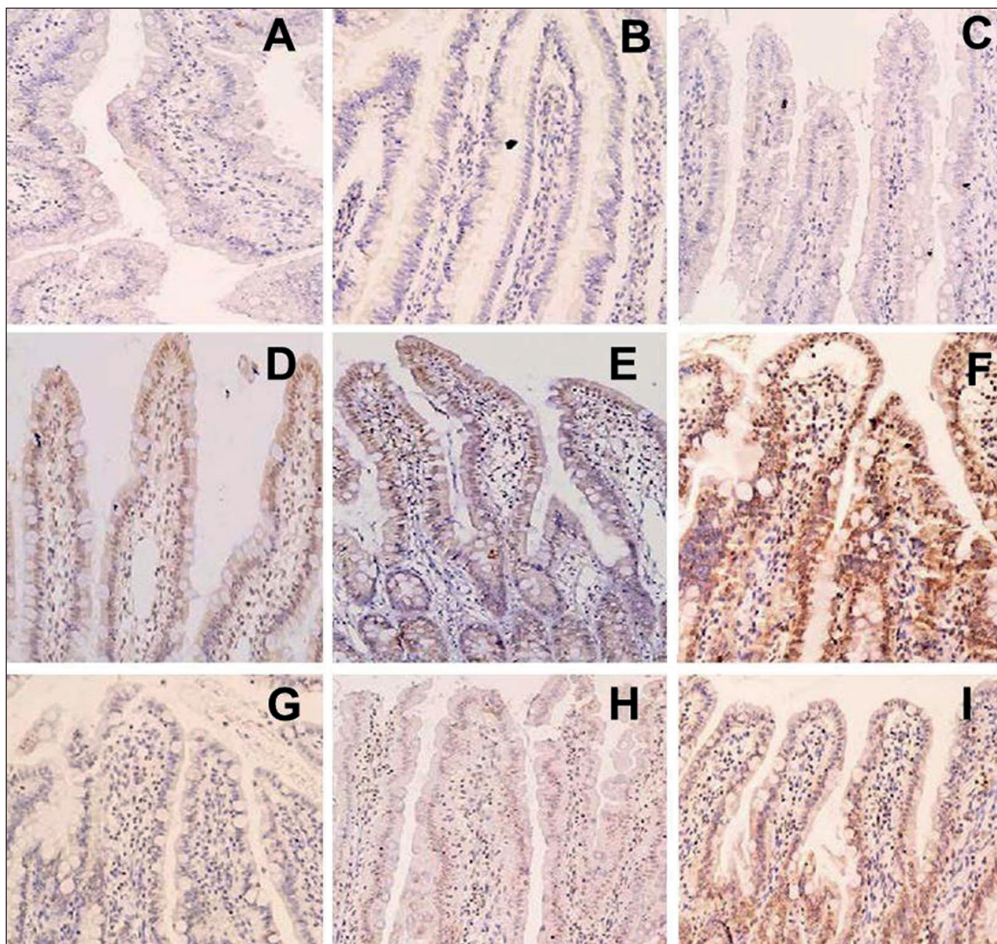


Figure 9. HMGB1 Immunohistochemical Staining (*200 magnification). HMGB1 staining were yellow or brownish yellow particles: the sham group on day 3 (A), the sham group on day 7 (B); the sham group on day 14 (C); the OJ group on day 3 (D), the OJ group on day 7 (E), the OJ group on day 14 (F), the OJPUFA group on day 3 (G), the OJPUFA group on day 7 (H), and the OJPUFA group on day 14 (I).

the OJPUFA group was mainly located in the nucleus and in the cytoplasm of inflammatory cells in the stroma (Figure 9H). On day 14, the expression of HMGB1 in the OJ group continued to increase substantially (Figure 9F). The expression of HMGB1 in the OJ group was stronger than that in the OJPUFA group (Figure 9I).

Results from Western-blot relative quantitative analyses were consistent with results from immunohistochemical staining (Figure 10A). HMGB1 expression in the sham group maintained at a low level. While the expression in the OJ group and the OJPUFA group significantly increased ($p < 0.05$ for both). Since day 3, the expression of HMGB1 in the small intestine in the OJ group and the OJPUFA group was significantly higher than that in the sham group. On day 14, the level of HMGB1 in the OJ group was significantly higher than that in the OJPUFA group ($p = 0.001$).

Similar trends of change were observed in TLR4 and NF- κ B p65 expression based on immunohistochemical staining (Figure 11 and Figure 12) and Western blot (Figure 10B and 10C). On day 14, the expression of TLR4 and NF- κ B p65 in the OJ group was significantly higher than that in the OJPUFA group ($p < 0.001$ for all).

mRNA Expression of HMGB1, TLR4, and NF- κ B p65

For each indicator, the mRNA expression level in the sham group at each time point was used as

the reference group, and relative expression levels of each gene in the OJ group and the OJPUFA group were calculated. Compared to the sham group, expression levels of the above genes in the OJ group and the OJPUFA group were significantly higher (Figure 13A, 13B, and 13C). On day 3, there was no significant difference in the expression level of each gene between the OJ group and the OJPUFA group. On day 7 and day 14, the expression level of HMGB1 mRNA in the OJ group was significantly higher than that in the OJPUFA group ($p = 0.010$ for day 7, and $p < 0.001$ for day 14). On day 14, the expression levels of TLR4 and NF- κ B p65 mRNA in the OJ group were significantly higher than those in the OJPUFA group ($p < 0.001$ for both).

Discussion

In summary, we successfully established an OJ rat model and collected lymph through a thoracic catheter. ω -3 PUFA improved the weight recovery and liver function of rats, reduced the release of pro-inflammatory factors including HMGB1, TNF- α and IL-1 β , lowered the excessive increase of IL-10 levels, and improved the immune disorder status of the OJ. At the same time, ω -3PUFA lowered the damage of intestinal villi and intestinal mucosal epithelium and improved the number and function of goblet cells in intestinal mucosal

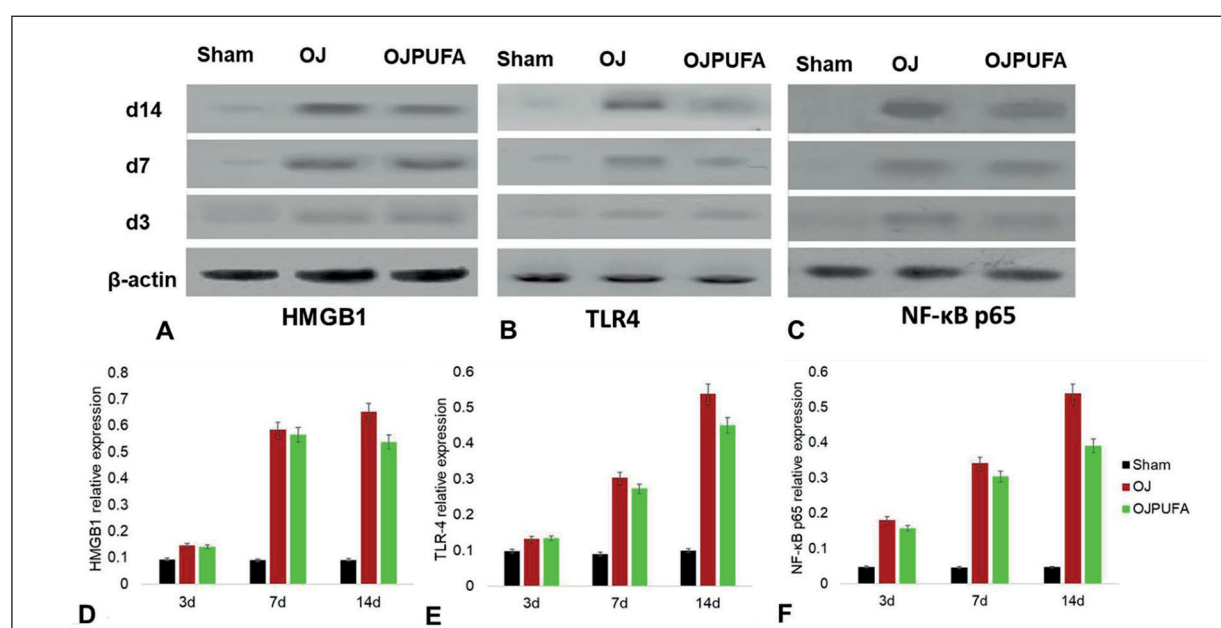


Figure 10. Western blot analyses of HMGB1 (A), TLR4 (B) and NF- κ B p65 (C).

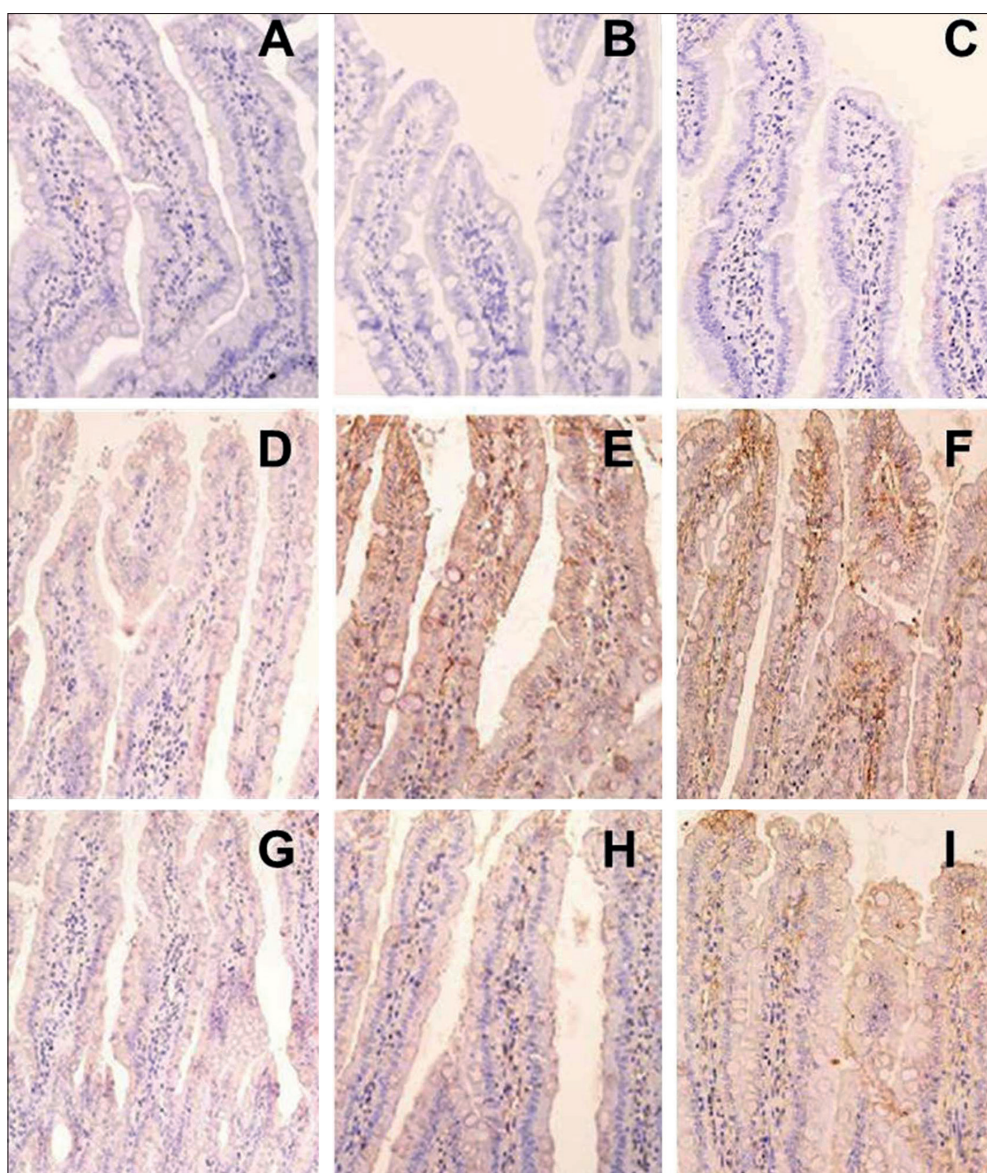


Figure 11. TLR4 immunohistochemical Staining (*200 magnification). TLR4 staining were yellow or brownish yellow particles: the sham group on day 3 (A), the shame group on day 7 (B); the shame group on day 14 (C); the OJ group on day 3 (D), the OJ group on day 7 (E), the OJ group on day 14 (F), the OJPUFA group on day 3 (G), the OJPUFA group on day 7 (H), and the OJPUFA group on day 14 (I).

epithelium. OJ can activate the HMGB1/TLR4/NF- κ B pathway in intestinal tissues and result in intestinal mucosal barrier dysfunction; ω -3 PUFA may exert protective effects on the intestinal mucosa by inhibiting this pathway.

OJ is a common disease in hepatobiliary surgery department. Although the biliary obstruction can be relieved by surgery, high incidence of multiple organ injuries during perioperative period and its associated mortality remains challenging in clinical practice³¹. Multiple organ injury is

closely related to intestinal mucosal barrier dysfunction, enteroendoxemia and bacterial translocation¹¹. In this study, we used common bile ducts ligation to establish obstructive model. The total serum bilirubin and direct bilirubin levels of rats reached a peak on day 3, as expected. Regarding the choice of the location of lymphatic catheterization, previous literature has reported using mesenteric lymphatic catheterization^{32,33}, cervical thoracic catheterization, and abdominal thoracic catheter lymphatic drainage. The volume of lym-

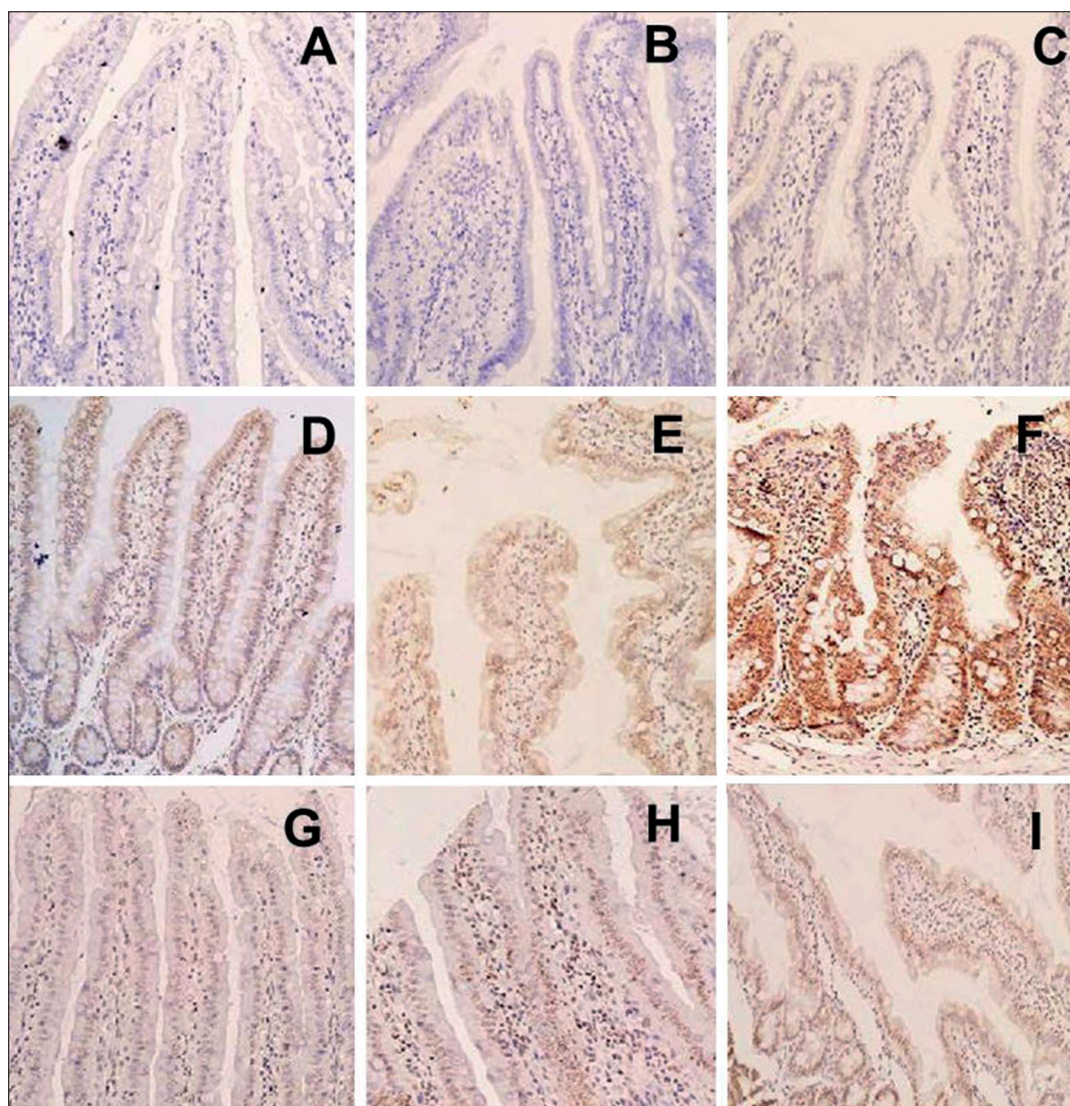


Figure 12. NF-kB p65 immunohistochemical Staining (*200 magnification). NF-kB p65 staining were yellow or brownish yellow particles: the sham group on day 3 (A), the sham group on day 7 (B); the sham group on day 14 (C); the OJ group on day 3 (D), the OJ group on day 7 (E), the OJ group on day 14 (F), the OJPUFA group on day 3 (G), the OJPUFA group on day 7 (H), and the OJPUFA group on day 14 (I).

phatic drainage of mesenteric lymphatic catheters is small, while the anatomy of cervical thoracic catheters is complicated and may result in long operation time. We used abdominal thoracic duct lymph drainage for the following reasons: the position of the abdominal thoracic duct is relatively constant; the drainage volume is large; and most of the lymph sources specifically come from the abdominal organs. Although the catheterization operation is relatively complicated, we separated and exposed the thoracic duct along the abdominal aorta. We achieved high success rate and large drainage volume in this project.

After obstructive jaundice, a large number of cytokines are produced and later enter the systemic circulation, which can induce a systemic inflammatory response⁹. After OJ, levels of inflammatory factors, including TNF- α and IL-1 β and anti-inflammatory factor IL-10 increased. We demonstrated that ω -3 PUFA is able to decrease the levels of above-mentioned factors. This is consistent with previous studies in rat models and *in vitro* culture of immune cells^{22,34}. In a recent randomized, controlled, double-blind clinical study of preterm infants (26-32 weeks), intravenous nutrition supplemented by ω -3 PU-

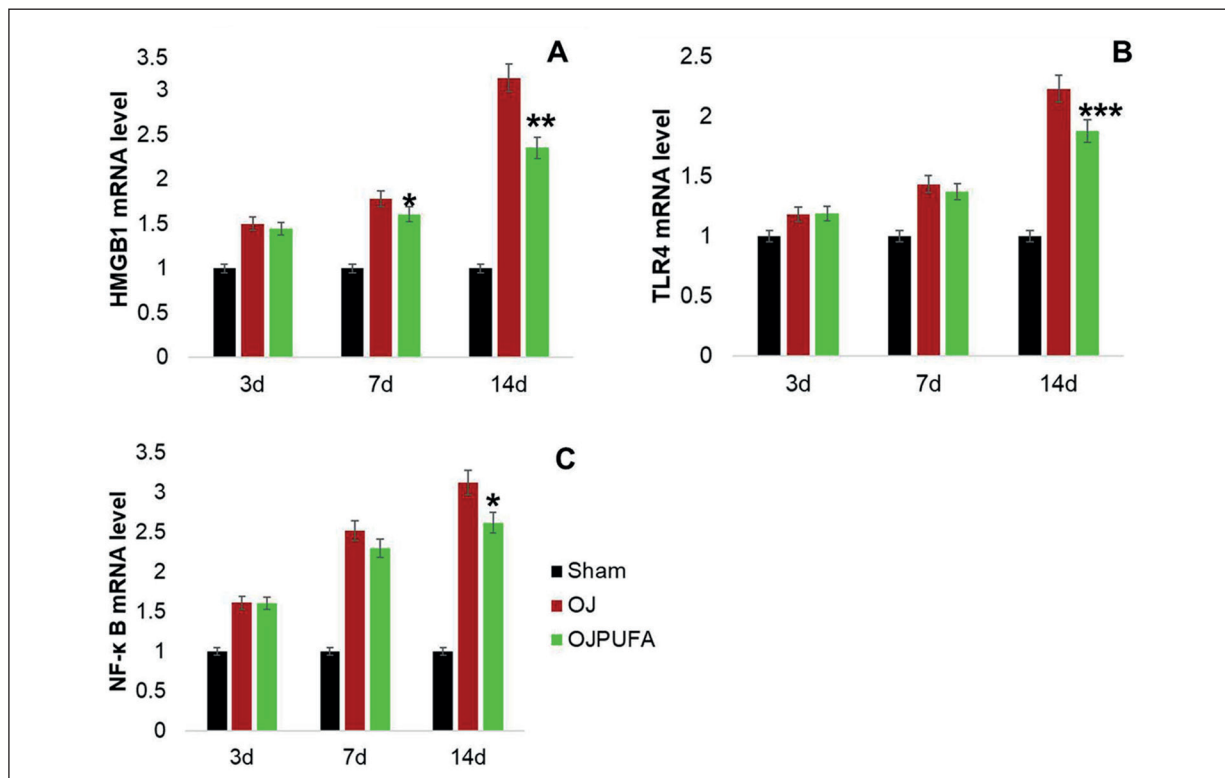


Figure 13. Relative mRNA expression of HMGB1 (A), TLR4 (B), and NF-κB (C) detected by qRT-PCR. The mRNA expression in the Sham group at the same time point was used as the reference group. * $p < 0.05$ compared with the OJ group; ** $p < 0.01$ compared with the OJ group; *** $p < 0.001$ compared with the OJ group.

FA can significantly reduce serum TNF- α , IL-6, and IL-8 levels²⁴.

Comparing the cytokine content in serum versus in lymph, we found that the concentrations of TNF- α and IL-1 β in lymph were higher than those in the serum, especially after the 7d time point. It is possible that in prolonged biliary obstruction, cytokines entered the systemic circulation through the “intestinal lymphatic” pathway. Previous studies^{32,35} have found that in animal models of abdominal infection, traumatic shock, and intestinal ischemia-reperfusion, endotoxin, bacteria, and other substances entered the systemic circulation by the “intestinal lymphatic” pathway. In severe abdominal infections, the density of fluorescent marked LPS was found to be highest in mesenteric lymph nodes and lymphatic pathway may be their main path to enter the systemic circulation³⁶. A recent study confirmed that TNF- α and IL-1 β increase the permeability of lymphatic endothelial cells, which in turn facilitate the absorbance of cytokines into the systemic circulation³⁷. However, the level of IL-10 in serum was significantly higher than that in the lymph, indi-

cating that lymph may not be the main source of IL-10 in OJ.

Wang et al³⁸ reported that LPS can stimulate the release of HMGB1 protein from mouse macrophages and plays an important role in the progress of sepsis as a late-stage inflammatory mediator. HMGB1 stimulates the production of endogenous inflammatory factors such as TNF- α and IL-1 β . In turn, these factors increase production of HMGB1 and form a cascade amplification effect³⁹. In our work, ω -3 PUFA reduced the secretion of HMGB1. Similar results were observed in small bowel transplantation⁴⁰ and partial hepatic ischemia-reperfusion model⁴¹. We found that serum level of HMGB1 on day 3 and day 7 were higher than those in the lymph, while lymphatic level of HMGB1 was higher than the serum level on day 14. It is speculated that in early OJ, HMGB1 is mainly produced by liver kupffer cells. However, in later stage, the gut-associated lymphoid tissue may be activated and HMGB1 production by these inflammatory cells increases and enter the systemic circulation through the lymphatic vessels. At the same time, serum NO

level is significantly higher than that in the lymph fluid. The main source of NO may be from macrophage systems from other organs, such as liver Kupffer cells⁴².

Taking all the changes of TNF- α , IL-1 β , IL-10, HMGB1, and NO together, it can be inferred that as the prolongation of biliary obstruction, the “intestinal lymphatic” pathway becomes more important and more inflammatory factors enter the systemic circulation through this pathway. This suggests that the “intestinal lymphoid” pathway plays an important role in the progression of obstructive jaundice. Even only small amounts of cytokines enter the systemic circulation can result in substantial damage due to the “cascade amplification” effect among cytokines. Altogether, the role of the lymphatic pathway cannot be ignored.

ω -3 PUFA showed a protective effect on the intestinal mucosa epithelium and intestinal villi structure and reduced changes in villus length/crypt depth ratio. This ratio reflects the degree of renewal and metabolism of the intestinal mucosal epithelial cells. A decrease in this ratio also indicates reduced small intestinal absorption area, which can aggravate malnutrition in obstructive jaundice. ω -3 PUFA significantly improved the ratio, indicating that ω -3 PUFA may have a protective effect on the cells with proliferation ability in the pit, and may promote its proliferation, differentiation and maturity. This is consistent with results of previous animal experiments⁴³. In addition, ω -3 PUFA significantly improves the number and quality of goblet cells in the intestinal epithelium and plays a protective role in maintaining the intestinal mucosal barrier.

HMGB1, as an endogenous ligand of TLR4, can activate NF- κ B and initiate the expression of inflammatory cytokines, thus plays an important role in the progress of inflammation and immune regulation^{44,45}. After OJ, the relative mRNA and protein expression of HMGB1, TLR4, and NF- κ B significantly increased. It suggests that the HMGB1/TLR4/NF- κ B pathways may be involved in the process of intestinal mucosal epithelial cells injury caused by OJ.

A previous study⁴⁶ suggested that HMGB1 can activate the TLR4 pathway, inhibit the migration and differentiation of intestinal epithelial cells, and impair the repair process of intestinal mucosal epithelial cells. This might be one reason for the decrease in the number of goblet cells in the OJ rats. The expression of HMGB1, TLR4 and NF- κ B in the OJPUFA group were lower than

those in the OJ group, suggesting that PUFA may reduce the damage to the intestinal mucosa by inhibiting the HMGB1/TLR4/NF- κ B pathway. Previous studies^{32,33,41} have shown that ω -3 PUFA can regulate the HMGB1/TLR4/NF- κ B pathway and have a protective effect in *in vitro* mononuclear cell culture, intestinal ischemia-reperfusion injury or liver ischemia-reperfusion injury. At the same time, our results also suggest that inhibiting or reducing the activation of the HMGB1/TLR4/NF- κ B pathway may provide new treatment strategy for intestinal mucosal barrier dysfunction caused by obstructive jaundice.

Conclusions

We demonstrated that ω -3 PUFA has a protective effect in management of obstructive jaundice. ω -3 PUFA can regulate the inflammatory response, reduce damage to intestinal structure, and improve the number and function of goblet cells in intestinal mucosal epithelium. Reducing the activation of HMGB1/TLR4/NF- κ B pathway may be one mechanism for ω -3 PUFA's protective effect. We suggested that ω -3 PUFA and HMGB1/TLR4/NF- κ B pathway might be a treatment strategy in obstructive jaundice.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethical Approval

All animal experiments in this study complied with the guidelines for animal experiment of the Ethics Committee of Binzhou Medical College.

Availability of Data and Material

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Authors' Contribution

Guarantor of integrity of the entire study: Xuting Zhi; study concepts: Xingyuan Zhang; study design: Changxi Zhang, Xuting Zhi; definition of intellectual content: Changxi Zhang, Xuting Zhi; literature research: Xutao Lin, Qinghai Guan; experimental studies: Chunmei Shu, Changxi Zhang, Fan Zhang; data acquisition: Fan Zhang, Changxi Zhang; data analysis: Chunmei Shu; statistical analysis: Qinghai Guan manuscript preparation: Changxi Zhang; manuscript editing: Changxi Zhang, Xingyuan Zhang; manuscript review: Xuting Zhi.

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