

Original Article

Adhesion of leukocytes is involved in increased β -endorphin and elevated pain threshold in obstructive jaundice

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Abstract: The elevated pain threshold is a special change in obstructive jaundice (OJ). It does not attract enough attention, which leads to over-use of opioid drugs and causes many peri-operative complications. In the present study, β -endorphin in the plasma of OJ patients was measured by ELISA. Leukocytes were isolated from peripheral blood of the OJ and non-jaundice (Non-J) patients. The mRNA of β -endorphin precursor proopiomelanocortin (POMC) and μ -opioid receptor (MOR) in the leukocytes of OJ patients were measured by Real-time PCR. After treated with bilirubin, the leukocytes from Non-J patients were used to measure the expression of μ -opioid receptor and β -endorphin. Opioid-containing leukocytes were also measured by Flow Cytometry after bilirubin incubation. After the obstructive jaundice model of rats was established by bile duct ligation (BDL), the rats were injected with ICAM-1 antibody. The pain threshold of rats was detected and β -endorphin in the plasma was measured by ELISA method. The results showed that β -endorphin was higher in the OJ patients than that in the Non-J patients. The mRNA of POMC and MOR in the leukocytes were significantly higher in the OJ patients than that in the Non-J patients. After leukocytes from Non-J patients were treated with bilirubin, β -endorphin in the supernatant was up-regulated. The POMC mRNA of leukocytes was also increased. Meanwhile, the number of opioid-containing leukocytes was significantly raised after bilirubin incubation. In the rat model of OJ, elevated pain threshold was reversed after treated with ICAM-1 antibody (0.5 mg/kg, 1 mg/kg). Meanwhile, the β -endorphin in the plasma was down-regulated by ICAM-1 antibody. The results demonstrated that the up-regulation of β -endorphin is probably related to the elevated pain threshold in OJ patients. The results further suggested that the adhesion of leukocytes was involved in the up-regulation of β -endorphin and MOR as well as elevated pain threshold among OJ patients. The changes in opioid-containing leukocytes are probably an alternative explanation to this special pain change in OJ patients.

Keywords: Leukocyte, β -endorphin, obstructive jaundice, pain threshold, bilirubin

Introduction

Obstructive jaundice (OJ), a common clinical manifestation, is frequently caused by gallstones and malignant tumors. Patients with OJ, especially for the "painless jaundice", have a markedly reduced pain perception [1]. During perioperative period, the relative lower opioid consumption caused by the elevated pain threshold leads to relative over-dosage of opioid drugs, which increases complications and mortality in OJ patients, such as delayed recovery and respiratory depression. However, this problem has not been given enough attention. In our previous studies, we have shown that the intraoperative requirement of remifentanyl in patients with OJ was significantly decreased

compared with that in controls without jaundice [2]. The electrical basal pain threshold was significantly higher and postoperative morphine consumption was lower in patients with jaundice [3]. The underlying mechanism needs further study.

β -endorphin is an endogenous analgesic neuropeptide, produced from processing of the precursor proopiomelanocortin (POMC). The increase of plasma β -endorphin in OJ patients has been proved and the plasma β -endorphin also tends to increase along with the rising of bilirubin. It is suggested that β -endorphin could be involved in the elevated pain threshold in jaundice patients. Liver dysfunction caused by jaundice may lead to the decrease of the

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metabolism of the endogenous β -endorphin, which results in the increase of β -endorphin. Evidence has suggested that endogenous opioid mediated antinociception in cholestatic mice due to the local effects of endogenous opioids at sensory nerve endings [4]. The causes of the increase of β -endorphin need further researches.

Patients with OJ show high level of peripheral leukocytes. Many researches have revealed that some leukocytes in peripheral blood could secrete opioid peptide, mainly β -endorphin and parts of dynorphin and enkephalin under certain pathophysiological conditions, such as jaundice [5, 6]. Various studies have shown that cyclophosphamide, cyclosporine A and other antineoplastic drugs compromised the analgesic effects in the elevated pain threshold models caused by stress due to the inhibition of the activation and adhesion of opioid-containing leukocytes [7-9]. Whether the opioid-containing leukocytes are involved in the increase of β -endorphin and the elevated pain threshold in OJ patients requires further studies.

In the present study, μ -opioid receptor and β -endorphin expression in the leukocytes of OJ patients were measured. The leukocytes from Non-J patients were treated with bilirubin and then the expression of μ -opioid receptor and β -endorphin were detected to investigate whether opioids in leukocytes were involved in the change of pain threshold in OJ patients. Meanwhile, ICAM-1 antibody were used in the rat model of obstructive jaundice to further investigate whether the adhesion of leukocytes was related to the up-regulation of β -endorphin and elevated pain threshold in OJ.

Patients and methods

Patients

The study was approved by the Institutional Ethics Committee (Eastern Hepatobiliary Surgery Hospital, Shanghai, China), and informed consent was obtained from each patient undergoing elective surgery for hepatobiliary diseases. Eighteen patients with obstructive jaundice (serum total bilirubin $>20 \mu\text{M}$) caused by neoplasm of the bile duct or the head of the pancreas were included in the study (OJ patients) [10]. Sixteen men with pancreas tumor and no jaundice (serum total bilirubin $<20 \mu\text{M}$) were included as control (non-J patients). All patients were rated status I or II according to the

American Society of Anesthesiologists physical (ASA) and were aged between 20 and 70 yr. Exclusion criteria were 1) age older than 70 yr or younger than 20 yr; 2) significant obesity (body mass index $>30 \text{ kg/m}^2$); 3) a history of diabetes mellitus, cardiovascular, respiratory, or renal disease; 4) hepatic encephalopathy, psychiatric illness, or neuropathy; 5) a history of acute or chronic pain; 6) a history of either alcohol or drug abuse; 7) a history of opioids application within a week. Peripheral blood was obtained before elective surgery for peripheral blood leukocytes.

Measurement of β -endorphin in plasma

β -endorphin level was measured using a Elisa β -endorphin Kit (Sigma, St. Louis, MO, USA) and resultant optical density was determined using a microplate reader of hermo Multiskan MK3 (Thermo Fisher Scientific, MA, USA) at 450 nm. It was performed according to the manufacturer's instructions [11]. Results were expressed as ng/mL.

Measurement of MOR mRNA and POMC mRNA expression in leukocytes

After low-speed centrifugation of peripheral blood for 15 min, blood cells were collected and red blood cells were damaged. Then cells in the leukocytes layer were collected and purified. The MOR mRNA and POMC mRNA in leukocytes were detected by Real time-PCR methods. The PCR primers of μ -receptor were as follows: 5'-GCCCTTCCAGAGTGTGAATTAC-3' (forward); 5'-GTGCAGAGGGTGAATATGCTG-3' (reverse). The PCR primers of POMC were as follows: 5'-CCCCTACAGGATGGAGCACTT-3' (forward); 5'-GATGGCGTTTTTGAACAGCGT-3' (reverse).

The Real-Time PCR Detection System (Roche, Switzerland) continually monitors the increase in fluorescence, which is directly proportional to the PCR product [12].

Opioid-containing leukocytes counted by flow cytometry

The leukocytes from non-J patients (n=6) were collected and incubated on 12-well plates. After the numbers of the cells were counted, bilirubin of $102 \mu\text{M}$ was added into each plate. The same volumes of PBS were added as control. After incubated with bilirubin for 24 h, the

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Table 1. Demographic data of the patients

	Non-J	OJ	P Value
N	16	18	
Age (y)	53.6±9.0	55.2±9.6	0.620
Weight (Kg)	63.2±4.7	64.1±10.3	0.734
Total bilirubin (μmol/l)	10.5±3.4	183.6±117.8	<0.01
Bile acids (μmol/l)	9.4±6.3	70.3±49.8	<0.01
Albumin (g/l)	42.4±2.5	36.6±3.9	<0.01
ALT (U/l)	26.8±14.5	143.0±85.3	<0.01

There was no difference in age, weight, and body mass index between two groups ($P>0.05$). Total bilirubin, bile acids, and alanine aminotransferase in plasma were significantly higher in OJ group compared to those in Non-J group ($P<0.01$). Obstructive jaundice was indicated as OJ; patients without jaundice were indicated as Non-J patients.

leukocytes were harvested and incubated with anti- μ receptor antibodies (1:20; Sigma, St. Louis, MO, USA) at room temperature for 30 minutes. The FITC-labeled secondary antibodies (1:300; Sigma, St. Louis, MO, USA) were then added and incubated for 1 h at room temperature. After supernatant and suspending cells were removed, 500 μ L of 1% paraformaldehyde was added and then measured by Flow Cytometry (Beckman Coulter Inc, BREA, CA, USA). β -endorphin in supernate was also measured by ELISA.

Animals

This study was approved by the Animal Care Committee of the Second Military Medical University and performed in accordance with the Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (weighing 200-250 g) were obtained from the Shanghai Slac Experimental Animal Centre (Shanghai, China). The rats were housed in individual cages in a temperature-controlled room with alternating 12 h light/dark cycles. Food was withheld 8 h before the starting of the experiments, but all animals had free access to water. Monitoring for health problems was performed three times a day and no death was found during the experiments. The rats were euthanized by CO_2 after the whole experiments were finished.

Grouping

SD rats were randomly divided into 4 groups: a sham group, a PBS group ($n=8$), an ICAM-1 0.5 mg/kg ($n=8$, intravenous injection of 0.5 mg/kg of ICAM-1 antibody) and an ICAM 1 mg/kg group ($n=8$, intravenous injection of 0.5 mg/kg of ICAM-1 antibody). On the 7th day after ligation,

0.5 mg/kg and 1 mg/kg ICAM-1 antibodies were injected intravenously in the ICAM-1 0.5 mg/kg group and ICAM-1 1 mg/kg group respectively after the measurement of pain threshold. The same volumes of PBS were injected in the PBS and Sham group as well. After 0.5, 1, 2 hours of injection, mechanical pain thresholds were detected using von Frey. The β -endorphin concentrations in the plasma were also measure by ELISA method.

Establishment of obstructive jaundice models

The obstructive jaundice model was established by bile duct ligation (BDL). After rats were anesthesia by the injection of chloral hydrate (300 mg/kg, i.p.), the main bile duct was ligated using two ligatures approximately 0.5 cm apart and then transected at the midpoint between the two ligatures. The bile duct was isolated without ligation in the sham rats after opening the abdomen. Cholestasis was confirmed by increased serum level of bilirubin as well as intact bile duct ligature and proximal dilation of the bile duct at the time of sacrifice [3].

Mechanical pain threshold testing

Mechanical pain threshold testing was performed using Electronic von Frey hair (IITC Life Science, Woodland Hills, CA, USA). Rats were acclimated daily for 10 min/day to the test environment (a Plexiglass box on a metal grid surface) for 3 days. On test days, rats were allowed to acclimate for 5-10 min. The nociceptive stimulus, a single rigid filament attached to a hand-held transducer, was applied perpendicularly to the medial surface of the hind paw with increasing force. The endpoint was taken as nocifensive paw withdrawal accompanied by head turning, biting and/or licking. As soon as this reaction occurred, the required pressure was indicated in grams, and this value was considered to be the individual paw withdrawal threshold (PWT) value [13]. Each rat was tested in triplicate per time point and the average for the three measurements was then calculated. Set 80 g as the maximum.

Statistical analysis

All the data were expressed as mean \pm standard deviation and analyzed by SPSS 17.0 sta-

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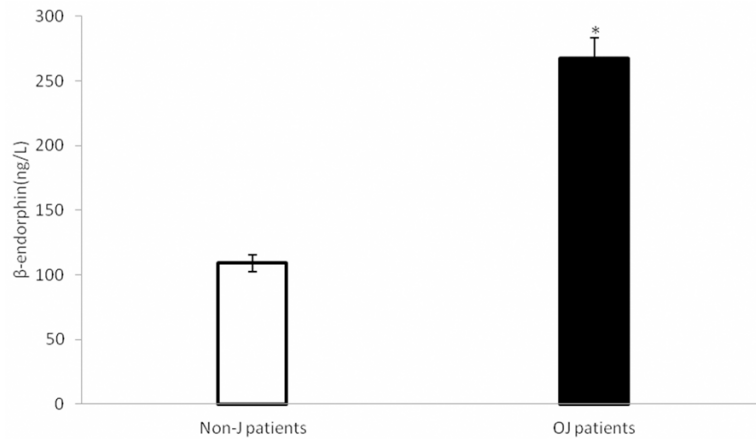


Figure 1. β-endorphin expression in plasma of patients. The plasma β-endorphin increased in the OJ patients compared to Non-J patients detected by ELISA assay (* $P<0.05$). Obstructive jaundice was indicated as OJ; patients without jaundice were indicated as Non-J patients.

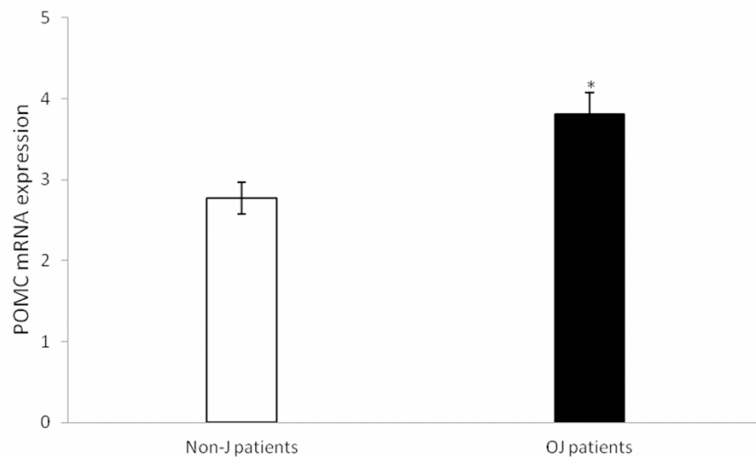


Figure 2. POMC mRNA of leukocytes from OJ patients. The results of real-time PCR showed that MOR mRNA in leukocytes increased significantly in the OJ patients compared to the Non-J patients (* $P<0.05$). Obstructive jaundice was indicated as OJ; patients without jaundice were indicated as Non-J patients; μ-opioid receptor was indicated as MOR.

tistical software. Statistical analyses were performed using one-way or two-way ANOVA. It was considered statistically significant when $P<0.05$.

Results

Demographic data

As shown in **Table 1**, there was no difference in age, weight, and body mass index between two groups ($P>0.05$). Serum concentrations of total bilirubin, bile acids, and alanine aminotransferase were significantly higher in the OJ group compared to the Non-OJ group ($P<0.01$).

Elevation of β-endorphin in plasma among OJ patients

The blood samples of OJ and Non-J patients were collected and β-endorphin in plasma was measured by ELISA. The results in **Figure 1** showed that plasma β-endorphin was markedly higher in the OJ group compared to the Non-J group ($P<0.05$). The results suggested that the elevated pain threshold in OJ patients might be caused by the elevation of β-endorphin.

Up-regulation of MOR and POMC mRNA in leukocytes of OJ patients

The MOR and POMC mRNA in leukocytes of OJ and Non-J patients were detected with real-time PCR method. As shown in **Figure 2**, MOR mRNA increased significantly in the OJ patients compared to the Non-J patients ($P<0.05$). Similarly, the mRNA of POMC was also higher in the OJ patients compared to the Non-J patients (**Figure 3**, $P<0.05$). The results suggested that the elevation of β-endorphin in the plasma could result from the high expression of POMC in the leukocytes. It also indicated that the elevated pain thresh-

old in OJ patients could relate to the up-regulation of MOR in leukocytes.

Changing of opioid system in leukocytes after bilirubin stimulation

To observe the changing of MORs and β-endorphin expression during jaundice, leukocytes from Non-J patients were incubated with bilirubin in vitro. The β-endorphin in the supernatants were increased after incubation with bilirubin for 24 h (**Figure 4**, $P<0.05$). Meanwhile, the POMC mRNA of leukocytes increased markedly (**Figure 5**, $P<0.05$). The results in **Figure 6** showed that the numbers of opioid-containing

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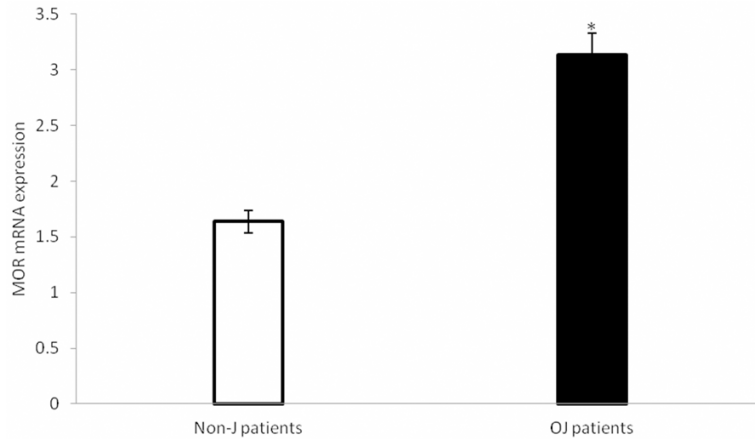


Figure 3. POMC mRNA of leukocytes from OJ patients. The POMC mRNA in leukocytes increased markedly in the OJ patients compared to the Non-J patients as detected by real-time PCR (* $P < 0.05$). Obstructive jaundice was indicated as OJ; patients without jaundice were indicated as Non-J patients; proopiomelanocortin was indicated as POMC.

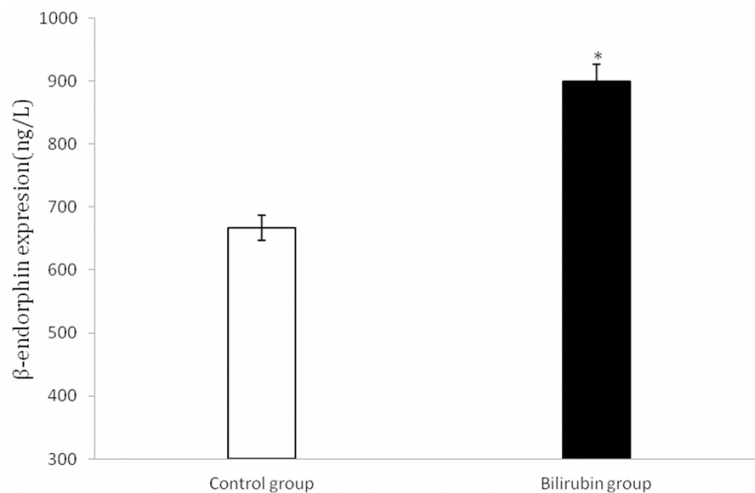


Figure 4. β -endorphin expression in the supernatant of leukocytes from OJ patients after bilirubin incubation. The levels of β -endorphin in the supernatant of the isolated leukocytes from Non-J patients were significantly increased after incubation with bilirubin for 24 h as detected by ELISA method. (* $P < 0.05$) Patients without jaundice were indicated as Non-J patients.

leukocytes were significantly increased after incubation with bilirubin (Ratio of opioid-containing leukocytes: control group $0.22 \pm 0.07\%$, bilirubin group $3.34 \pm 0.92\%$). The results suggested that bilirubin be related to the increase of opioid receptors and β -endorphin in leukocytes of OJ patients.

Decrease of pain thresholds after ICAM-1 antibody treatment

Compared to the sham group, mechanical pain thresholds increased in three groups receiving

BDL (Figure 7, $P < 0.05$). There was no difference in mechanical pain thresholds in three BDL groups ($P > 0.05$) at 0.5 h. After 1 h and 2 h of treatment, mechanical pain thresholds significantly decreased in the ICAM-1 0.5 mg/kg group and ICAM 1 mg/kg group compared to those of the PBS group ($P < 0.05$). The results indicated that the leukocytes adhesion could be involved in the elevation of pain thresholds.

Down-regulation of beta-endorphin after ICAM-1 antibody treatment

After 2 h of treatment with ICAM-1 antibody, β -endorphin concentrations in the plasma were markedly decreased in the ICAM-1 0.5 mg/kg group and ICAM 1 mg/kg group as compared to those in the PBS group (Figure 8, $P < 0.05$). It revealed that the leukocytes adhesion could lead to the up-regulation of β -endorphin in OJ state.

Discussion

The elevated pain threshold is a special change in obstructive jaundice with unknown mechanism. In the current study, β -endorphin in the plasma as well as the mRNA of POMC and MOR in the leukocytes were found increased

in the OJ patients. After leukocytes from Non-J patients were treated with bilirubin, β -endorphin level in the supernatant was up-regulated. The POMC mRNA of leukocytes was also increased. Meanwhile, the numbers of opioid-containing leukocytes were significantly increased after bilirubin incubation. In the OJ rats treated with ICAM-1 antibodies, the elevated pain thresholds were reversed along with the down-regulation of β -endorphin in the plasma. The results demonstrated that the up-regulation of β -endorphin and MOR in the leukocytes could be involved in the elevated pain threshold in OJ.

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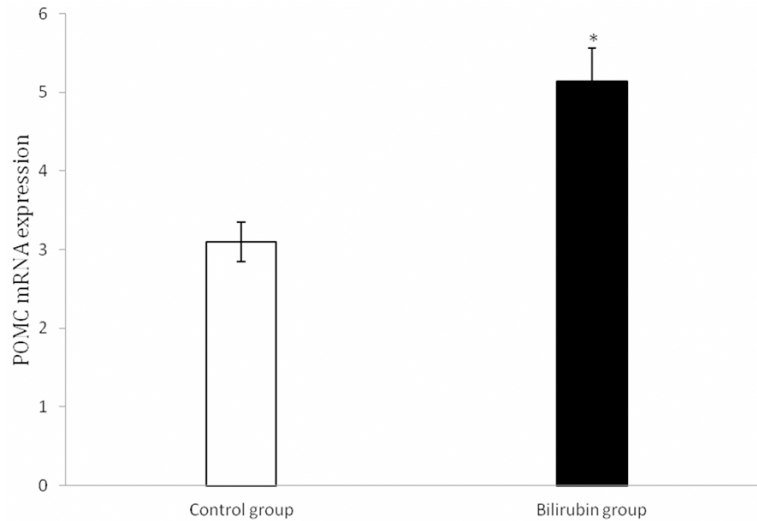


Figure 5. POMC mRNA of leukocytes from Non-J patients after bilirubin incubation. The POMC mRNA of leukocytes from Non-J patients increased markedly after bilirubin incubation as detected by real-time PCR (* $P < 0.05$). Obstructive jaundice was indicated as OJ; patients without jaundice were indicated as Non-J patients; proopiomelanocortin was indicated as POMC.

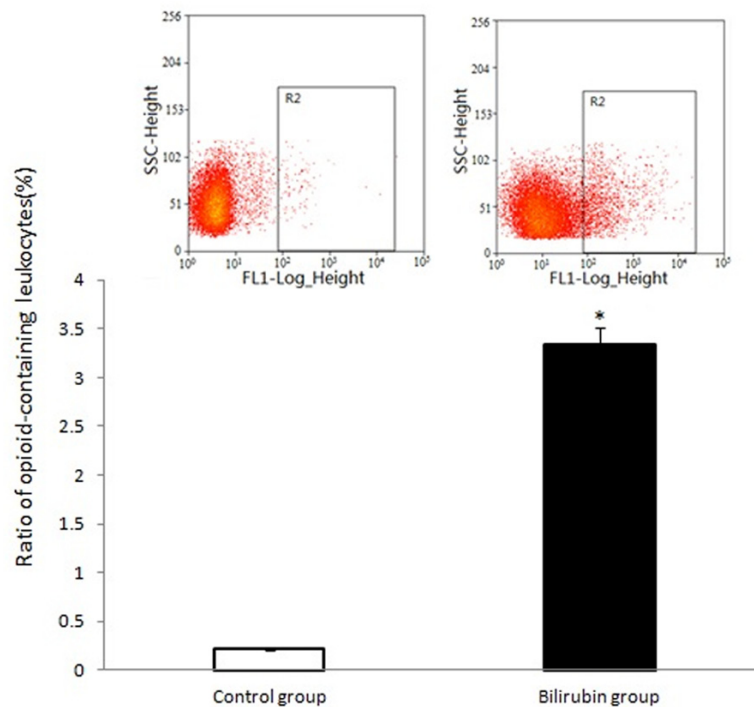


Figure 6. Detection of opioid-containing leukocytes from Non-J patients after bilirubin incubation. After incubation with different concentrations of bilirubin in (102 μ M) for 24 h, the results of FCM showed that the numbers of opioid-containing leukocytes from Non-J patients in the bilirubin group were significantly increased compared to that in the control group (* $P < 0.05$). The ratio of opioid-containing leukocytes/total leukocytes was increased significantly (Ratio of opioid-containing leukocytes: control group $0.22 \pm 0.07\%$, bilirubin group $3.34 \pm 0.92\%$). Patients without jaundice were indicated as Non-J patients.

The adhesion of leukocytes could be probably related to the up-regulation of β -endorphin and MOR as well as the elevated pain threshold in OJ.

The increase of endogenous opioids has been proved in OJ, which induces pruritus, hyporesponsiveness, hepatic encephalopathy and other symptoms [14, 15]. The up-regulation of enkephalin in keratinocytes of patients with OJ has been confirmed in our previous study and could be relevant to increased pain thresholds [3]. The increase of β -endorphin in the plasma of OJ patients and rats was also found in the present study, which was in agreement with the study that the state of antinociception was selectively reversed by opioid antagonist naloxone in rats with cholestasis [16]. We further demonstrated that POMC mRNA in the peripheral leukocytes were increased, which suggested that the increased β -endorphin could originate from leukocytes. Patients with OJ were mostly accompanied with systemic inflammatory response, which increased the numbers of leukocytes in jaundice patients. The opioid-rich leukocytes are the source of endogenous opioid and play a key role in peripheral antinociception [17]. The leukocyte-derived β -endorphin as well as enkephalin, dynorphin and endomorphin are important in peripheral antinociception [18-20]. Thus, β -endorphin from the peripheral leukocytes seems to be involved in the elevated

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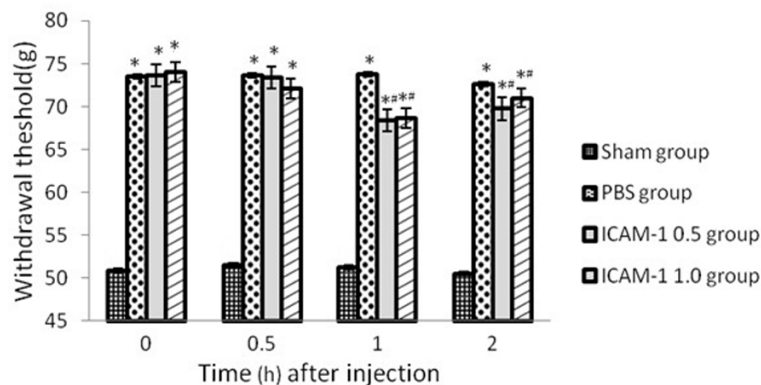


Figure 7. Mechanical pain thresholds after ICAM-1 antibody treatment. Mechanical pain thresholds of rats were measured by VonFrey. Compared to the sham group, mechanical pain thresholds increased in the three groups receiving BDL ($*P<0.05$). No difference was found in the ICAM-1 0.5 mg/kg group and ICAM 1 mg/kg group as compared to that in the PBS group 0.5 h after ICAM-1 antibody treatment ($P>0.05$). After 1 h and 2 h of treatment, mechanical pain thresholds significantly decreased in the ICAM-1 0.5 mg/kg group and ICAM 1 mg/kg group compared to those in the PBS group ($\#P<0.05$). Intercellular Adhesion Molecule 1 was indicated as ICAM-1.

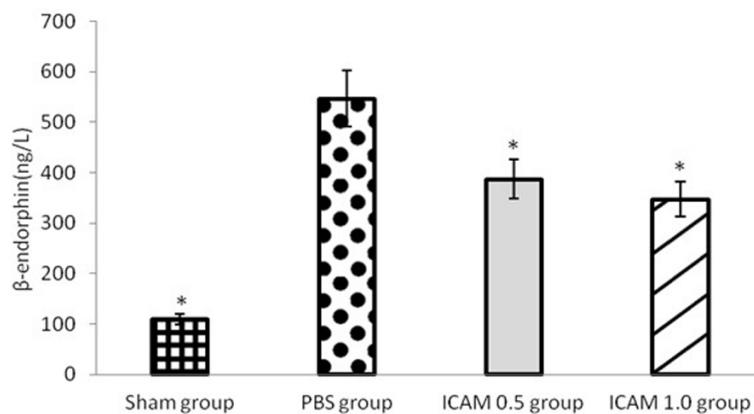


Figure 8. β -endorphin in plasma after ICAM-1 antibody treatment. After 2 h of treatment with ICAM-1 antibodies, β -endorphin in rats plasma were markedly decreased in the ICAM-1 0.5 mg/kg group and ICAM 1 mg/kg group as compared to that in the PBS group ($*P<0.05$). Intercellular Adhesion Molecule 1 was indicated as ICAM-1.

pain in OJ, which was also supported by Lisa Nelson [4] that endogenous opioid-mediated antinociception in cholestatic mice was peripherally mediated.

The peripheral antinociception effect of opioid-rich leukocytes does not depend on the release of opioids into the plasma [21]. The opioid-rich leukocytes were recruited surrounding the sensory nerve and adhered to the endings of sensory nerve through adhesion molecules such as ICAM-1, integrin [22-24]. Through this

way, opioids directly acted on opioid receptors to exert peripheral antinociception effect [25, 26]. ICAM-1 mediates the combination of the opioid-containing leukocytes and endothelial cells, which accelerates leukocytes accumulation and β -endorphin production. Previous studies revealed that inhibition of ICAM-1 by ICAM-1 antibody eliminated the analgesic effect of endogenous opioids through reducing the numbers of opioid-rich leukocytes and/or inhibiting the adhesion of the leukocytes with sensory nerves [27-29]. The current study demonstrated that the elevated pain thresholds in the OJ rats were reversed by ICAM-1 antibodies, which indicated that leukocytes adhesion to sensory nerve could be important in pain thresholds change in OJ. In inflammatory state, the up-regulation of endogenous opioids, such as β -endorphin, could increase the expression of inflammatory factors and then activate inflammatory cells [5, 30], which played a role of positive feedback. In this research, the decrease of β -endorphin in the plasma by ICAM-1 antibodies could also reduce the inflammatory response and reduce pain thresholds.

The pathogenesis of elevated pain in OJ is still unknown. It has been suggested that the symptom could arise as a result of an interaction between nerve endings and substances retained in the plasma [31]. In this context, bile acids have been considered as candidates [32]. Previous studies have revealed that bile acid induced analgesia through the activation of TGR5 receptors, a G protein-coupled plasma membrane receptor for bile acids [1]. In the current study, bilirubin was considered to be a candidate, since cholestatic patients showed

increased bilirubin concentrations in the plasma [33]. The hypothesis was supported by the results that intrathecal injection of bilirubin induced an anti-nociception effect in rats (unpublished data) and that bilirubin modulated the currents of neurons in a concentration-dependent manner [34]. The *in vitro* study showed that POMC mRNA of leukocytes and opioid-containing leukocytes increased after bilirubin incubation, which suggested bilirubin as an alternative potential mediator.

Conclusion

In conclusion, this study demonstrated β -endorphin and MOR were up-regulated in OJ patients. Inhibition of adhesion of leukocytes reversed the elevated pain threshold along with the down-regulation of β -endorphin and MOR caused by OJ. Leukocytes adhesion could probably be related to the up-regulation of β -endorphin and MOR, and further resulted in elevation of pain threshold in OJ patients.

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Disclosure of conflict of interest

None.

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