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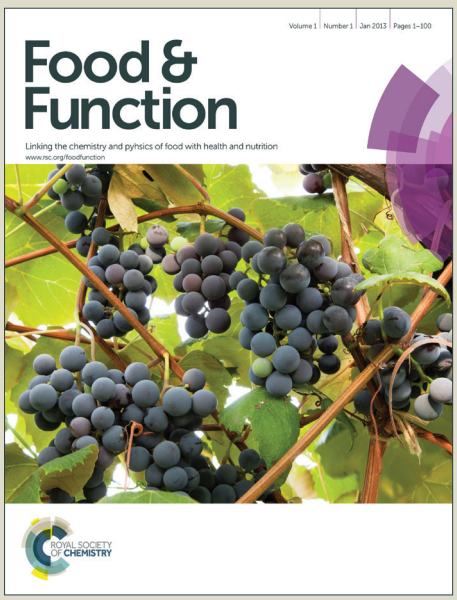

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The effect of curcumin on liver fibrosis in the rat model of microsurgical cholestasis

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Abstract

We aimed to determine the effects of curcumin on liver fibrosis and to clarify the role of nuclear factor-κB (NF-κB) and inducible nitric oxide synthase (iNOS) in the model of microsurgical cholestasis in early stage of extrahepatal biliary atresia. 12-week-old Wistar rats were divided into four groups (n=8): sham operated rats (received olive oil after laparotomy); curcumin group (received curcumin, 200mg/kg/day, after laparotomy); biliary duct ligated group (BDL, received olive oil after operation); biliary duct ligated group (BDL curc, received curcumin, 200mg/kg/day, after operation). After 3 weeks of the treatment, curcumin did not modify blood plasma markers as well as iNOS and NF-κB (p65) expressions in the liver species of the sham group. Interestingly, there was significant increase in both liver and kidney fibrosis extents. On the other hand, despite decrease in iNOS and NF-κB (p65) expressions, curcumin treatment did not affect fibrosis enlargement due to bile duct ligation in the liver. In the BDL group, curcumin treatment decreased the level of blood plasma markers investigated. In conclusion, curcumin treatment was able to improve functional properties of hepatocytes and to inhibit both NF-κB and iNOS upregulations in the BDL group, yet without beneficial effect on the liver fibrosis developed in this model of cholestasis. Thus, in the studied model of microsurgical cholestasis, other factors, different from NF-κB and iNOS are responsible for fibrotic process in the liver.

Key words: Curcumin, Microsurgical cholestasis, Liver fibrosis, NF-κB, iNOS

Introduction

Biliary atresia (BA) is the most common cause of pathologic jaundice in children and the most frequent indication for liver transplantation in the pediatric population worldwide [1]. It results from a progressive fibrosing and inflammatory cholangiopathy that begins in early infancy and rapidly progresses to the complete obstruction of the extrahepatic bile ducts [2]. BA is rather the phenotype of several disorders to which the infant liver responds in a stereotypic manner. The complex dynamic series of responses include inflammation, bile duct proliferation, apoptosis, and fibrogenesis [3-6]. Biliary atresia is the most rapid fibrosing liver disease in man, however despite numerous studies, the pathogenesis of liver fibrosis is not completely understood yet. The imbalance between proliferation and apoptosis of the hepatic stellate cells may represent one of the main causes of this process [7].

Since IFNγ plays such a dominant regulatory role in duct obstruction, it is possible that other proinflammatory cytokines such as IL-2, IL-12, TNFα and other soluble mediators, e.g. inducible nitric oxide synthase (iNOS) work in synergism to drive the pathogenesis of biliary atresia[2]. In this context, increased activity of nuclear factor-κB (NF-κB) has been shown after rotavirus challenge in neonatal mice. Blocking of NF-κB activity decreased inflammation in the liver and extrahepatic bile ducts [8]. Curcumin, the most important fraction of turmeric, has been shown to inhibit the activity of NF-κB [9], as well as diminish NF-κB subunit expression in db/db mice [10]. Recently, lowering effect of curcumin on serum level and gene expression of IL-2 and IL-6 has been observed in the rat model of hepatoxicity [11].

Curcumin has been found to have a number of pharmacological properties including antioxidant activity, induction of tumor cell apoptosis, protection against lipid peroxidation, suppression of protein kinase activation, or reduction of metal proteinase expression [12,13]. Based on these properties, a number of studies documented that curcumin exhibits anti-inflammatory [14,15], antioxidant [16-18], anticarcinogenic [19] and antimicrobial activities [20]. Moreover, curcumin has a variety of potentially therapeutic properties such as antineoplastic, antiangiogenic, antithrombotic, wound healing, antidiabetogenic, antistressor and antilithogenic actions [21,22]. Among the numerous targets that are responsible for beneficial effects of curcumin is modulation of transcription factor NF-κB and NF-κB-regulated gene products such as iNOS, cyclo-oxygenase-2, cyclinD1, adhesion molecules, MMPs, Bcl-2, Bcl-XL and TNF[23]. Curcumin has been further shown to abolish the phosphorylation and degradation of NFκB, which is a factor whose expression is induced by TNF, indicating that the step in the signal transduction pathway of NF-κB activation inhibited by this agent coincides with or precedes the phosphorylation step of NF-κB [24]. The aim of our study was to find out whether treatment with curcumin is able to decrease liver fibrosis induced by cholestasis and to clarify the role of NF-κB and iNOS in the model of microsurgical cholestasis in early stage of extrahepatal biliary atresia.

Materials and Methods

Chemicals

All the chemicals used were purchased from Sigma Chemicals Co. (Germany) if not specified. Curcumin was mixed as a suspension with olive oil and was applied via gavage in the dose 200 mg/kg from the first post operation day for 3 weeks daily.

Animals and treatment

All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Normal and Pathological Physiology SAS, and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use.

12-week-old male Wistar rats were randomly divided into four groups (n=8 in each group): sham operated rats, which received olive oil via gavage for 3 weeks after laparotomy; curcumin group, which received curcumin (200 mg/kg/day) in olive oil via gavage for 3 weeks after laparotomy; biliary duct ligated group (BDL), which received olive oil via gavage for 3 weeks after operation; biliary duct ligated group (BDL curc), which received curcumin (200 mg/kg/day) in olive oil via gavage for 3 weeks after operation.

All animals were housed at a temperature of 22–24 °C in individual cages and fed with a regular pellet diet ad libitum. Drinking water was supplied ad libitum. After 3 weeks of treatment the animals were sacrificed and the liver, kidney and blood samples were collected for biochemical, histological and immunohistochemical analyses.

Microsurgical experimental cholestasis

The experimental animals were anesthetized with Tiletamine –Zolazepam - Butorphanol (Zoletil®, VIRBAC, French, Butomidor, RICHTER PHARMA, Austria) combination

applied intraperitoneally. After the standard preparation of surgical field, upper midline laparotomy was performed. Microsurgical bile duct ligation was performed under Carl-Zeiss microsurgical microscope by 8-0 polypropylene ligatures. Muscles and skin were sutured by absorbable sutures in layers. In shamed operated animals, only upper midline laparotomy and subsequent wound closure were performed. For dehydratation and hypoglycaemia prevention warm saline (37^{o}C) including glucose was applied perioperatively to all animals. Cefadroxil (Ceporex, SCHERING PLOUGH, Ireland) and Butorphanol as antibiotic and anti-postoperative analgesia were administered subcutaneously for 3 subsequent days.

Blood biochemistry

Blood was collected into heparin tubes and then centrifuged. Following parameters were estimated in the blood plasma according to the kit instructions: alkaline phosphatase and aspartate aminotransferase activities, total protein, albumin, total bilirubin and conjugated bilirubin concentrations. Albumin/globulin ratio was calculated for all groups.

Histological examination of liver and kidney specimens

The liver and kidney samples were fixed in 10% formalin solution at room temperature and processed for light microscopy examination. Tissue sections embedded in paraffin were stained with hematoxylin–eosin (H&E) and picrosirius red, and thereafter examined and scored by pathologist blind to the treatment protocol. Histopathological evaluation was performed in four sections per slide for all specimens.

Histological changes of liver tissue were observed by polarized light microscopy and fibrosis enlargements were expressed as the area of positive signal per high-power field by ImageJ morphometric software. Ten fields of vision were selected randomly for every section, and the color image analysis system was used to calculate the ratio of positive expression area to the total field.

Immunohistochemical analysis

Expression of NF-κB (p65) and iNOS was detected by immunohistochemical staining in microarrays created from fixed liver tissues. Four cores with diameter 3 mm were randomly harvested from the tissue slices and paraffin embedded microarray was created. Sections (3 μm) were deparaffinized and rehydrated in phosphate buffered saline (PBS). Before immunohistochemical analysis, microwave epitope retrieval was performed (10 mM citrate buffer, pH 6.0; 20 minutes at subboiling temperature). The slides were subsequently incubated overnight in rabbit anti-iNOS (Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:100 and rabbit anti-p65 NF-κB (Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:100. The antibodies were diluted in Dako Real antibody diluent (Dako, Glostrup, Denmark). After 3 rinsing steps of 5 minutes each in PBS, sections were incubated for 30 minutes with histofine anti mouse and rabbit resp. anti goat antibody polymer conjugated with horse radish peroxidase (Nichirei Biosciences, Tokyo, Japan). After 3 rinsing steps of 5 minutes each in PBS, the peroxidase activity was visualized with diaminobenzidine (DAB, Dako, Glostrup, Denmark). Subsequently the sections were counterstained with hematoxylin. The positivity of each protein was evaluated in light microscope (Jenamed 2, Carl-Zeiss, Germany) and the localization of positivity was

determined. In cases of cytoplasmic positivity, the intensity was evaluated by morphometry using the ImageJ morphometric software v.1.33 (National Institutes of Health, USA). Results are expressed as ratio of area of protein positivity (characteristic brown color in DAB staining) to the total tissue area excluding the extracellular spaces.

Statistical analysis

Data are expressed as mean ± SEM. One-way ANOVA and Duncan`s test were used for analysis. Values were considered significant at $p<0.05$.

Results

Blood biochemistry

Concentrations of total protein, albumin, creatinine and urea as well as albumin/globulin ratio (A/G) are shown in Tab. 1. The activities of alkaline phosphatase (ALP) and aspartat aminotransferase (AST) and concentrations of total and conjugated bilirubin are presented in Fig. 1 and 2. Curcumin treatment did not affect these blood plasma markers of sham operated rats. Concentration of total protein was significantly elevated in BDL rats without albumin elevation. A/G ratio was 1.77 in sham operated rats, while in BDL rats the ratio decreased to 1.10 documenting the process of liver injury. The ratio was 1.21 in curcumin treated rats (Tab. 1). Plasmatic levels of AST and ALP, indicators of liver injury, increased about 2-fold after bile duct ligation when compared to the values of sham operated rats (Fig. 1). Total bilirubin and conjugated bilirubin concentrations were highly increased in BDL rats as a consequence of liver damage, cholestasis and

reduced conjugation ability of hepatocytes to bilirubin (Fig. 2). Pharmacological treatment with curcumin partially but significantly ($P<0.05$) prevented the increase of these blood plasma markers levels (Fig. 1, 2). Creatinine and urea concentrations were monitored as markers of renal function. Creatinine plasma level was not significantly changed in either group. In contrast, blood urea concentration was significantly elevated in BDL rats. This concentration was found to be on the control level in the curcumin treated animals (Tab. 1).

Liver and kidney fibrosis

As it is documented in the Fig. 3, chronic liver damage in BDL rats was accompanied by extended necrotic areas as compared with sham operated animals. Treatment of the BDL rats with curcumin did not result in significant differences in liver fibrosis enlargements. Morphometric measurement of collagen on PSR stained slides showed small and insignificant decrease of collagen level between BDL and curcumin treated BDL rats. In contrast, curcumin treated rats revealed significantly higher concentration of collagen compared to the sham group (Figs. 3,4). In the kidney, the similar changes of collagen level were determined (Fig. 4).

Expression of NF-κB (p65) and iNOS

Inducible NOS and NF-κB (p65) expressions in the liver of sham operated rats remained within control values despite curcumin treatment. In BDL rats, expressions of NF-κB (p65) and iNOS were increased about 10-fold and there were marked reduction in expression of both parameters in curcumin treated BDL rats (Figs. 5,6,7).

Discussion

In the present study, the effects of chronic treatment with curcumin on male Wistar rats subjected to bile duct ligation and sham operated rats with upper midline laparotomy were determined. Curcumin treatment did modify nor blood plasma markers studied neither iNOS and NF-κB (p65) expressions in the liver species of sham operated animals. Interestingly, there was significant increase in both liver and kidney fibrosis extents. On the other hand, despite decrease in iNOS and NF-κB (p65) expressions, curcumin treatment did not affect fibrosis enlargement elevated due to bile duct ligation in the liver. Because of the fact that the first months of the life of children with extrahepatal biliary atresia, one of the most important factors of liver destruction, is accompanied by cholestasis, we decided to use animal model of obstructive jaundice for investigation [2]. Several surgical techniques for developing extrahepatic cholestasis have been described, especially in the rat, based on the section of the bile duct between ligatures [25,26]. These techniques represent models of reversible obstructive jaundice, since they imply a high incidence of recanalization of the extrahepatic biliary route, which can be avoided by placing the duodenum and the distal part of the stomach between two ligated and sectioned ends of the bile duct [26]. The macrosurgical techniques of extrahepatic cholestasis, common bile duct ligation, caused development of infected hilar biliary pseudocysts by dilation of the proximal end of the bile duct. As a result, the animals died during the first 2 weeks of the postoperative period due to sepsis caused by multiple abscesses in the intraperitoneal, hepatic and pulmonary areas [27-29].

An advantage of the microsurgical technique of extrahepatic cholestasis in the rat is the absence of large biliary pseudocyst formation, which would explain why early mortality is not present. It is possible that the absence of the hilus pseudocyst in this microsurgical model of cholestasis decreases the incidence of hepatopulmonary infection and thus prevents mortality related to sepsis [29]. This was the reason why the microsurgical technique of extrahepatic cholestasis was used in our experimental model. Indeed, in our postoperative conditions none of the animals died.

Curcumin dose in our experimental model (200 mg/kg/day) was used on the base of previously reported publications, where the similar dose of curcumin successfully prevented liver injury induced by various hepatotoxic agens [30,31] or by chemically induced cholestasis [32].

Activities of ALP and AST as markers of liver injury, and levels of total protein, albumin and total and conjugated bilirubin as markers of hepatocyte function were determined. Albumin/globulin ratio was also calculated as parameter reflecting globulin overproduction and albumin underproduction. In our experimental conditions of microsurgical cholestasis, in early stage of extrahepatal biliary atresia, ALP and AST activities, total protein, A/G ratio as well as total and conjugated bilirubin were elevated significantly documenting liver injury and impaired function of hepatocytes. Creatinine and urea levels were monitored as markers of renal function. Creatinine plasma level was not significantly changed in either group. In contrast, blood urea concentration was significantly elevated in BDL rats as a symptom of protein catabolism. This concentration was found to be on control level in curcumin treated BDL animals. Curcumin treatment decreased also blood plasma activities of ALP and AST, total and

conjugated bilirubin, and A/G ratio which confirmed protective effect of curcumin in hepatocytes in obstructive cholestatic disease. Decreased level of blood urea in curcumin treated BDL rats was probably the result of decreased protein catabolism due to stabilization of liver function.

Cholestasis is defined as an impairment of bile flow through the liver. As a consequence, high concentrations of bile acids accumulate within the hepatocytes, causing tissue damage and liver failure. Several studies demonstrated that one of the mechanisms by which bile acids, especially hydrophobic bile acids, induce liver damage is by triggering hepatocyte apoptosis. Indeed, hydrophobic bile acids, such as deoxycholic and glycodeoxycholic acids, are able to cause hepatocyte apoptosis *in vitro* [33-35]. More remarkably, massive hepatocyte apoptosis is clearly detectable in the liver of bile duct-ligated mice, an animal model of extrahepatic cholestasis [36]. In addition, recent studies demonstrated that in a model of chronic cholestasis, Fas-mediated cytoxicity promotes development of liver fibrosis, the result of excessive deposition of extracellular matrix during the wound-healing response that follows a prolonged injury to the liver [37]. The increased expression of Fas and FasL may result from TNF-α-induced activation of NF-κB, a transcription factor that upregulates both these genes [38]. Since NF-κB upregulates iNOS - other inflammatory marker, we have decided to determine both p65 subunits of NF-κB and iNOS expressions in the liver species. In our experimental conditions of microsurgical cholestasis both p65 subunit of NF-κB and iNOS expressions were increased significantly.

Numerous studies, however, have shown that NF-κB provides survival signals in the context of death receptor-induced apoptosis in the liver. This process was assumed to

involve the transcriptional induction of various apoptotic suppressors [39]. Evidence that NF-κB governs critical anti-apoptotic proteins comes from well-described animal models. Injection of TNF into mice and addition of TNF to hepatic cells resulted in the activation of NF-κB [40]. Specific blockade of NF-κB activation by adenoviral-directed overexpression of the NF-κB inhibitor IκB significantly enhanced TNF-mediated apoptosis of hepatocytes [41]. A similar result was obtained by treatment of hepatocytes with the proteasome inhibitor lactacystin, which prevents degradation of IκBs [42]. Thus, in our experimental conditions, inhibition of NF-κB activity by curcumin treatment in sham operated rats led probably to impaired survival signals followed finally by fibrosis enlargement documented in both liver and kidney. Moreover, curcumin treatment significantly reduced NF-κB (p65) expression in BDL rats which may lead to reduced hepatocyte protection from apoptosis during liver regeneration. Curcumin also inhibited cell cycle progression during normal liver regeneration in rats, predominantly at the level of the G2/M transition point [43]. These processes could be associated with liver fibrosis. However, according to the Seehofer *et al* [43] the total liver mass and function might not be significantly altered, which corresponds well with our results. Additionally, curcumin treatment was not able to decrease fibrosis enlargement despite decreased iNOS expression. Marra *et al* [44] drew attention to the fact that hepatocytes are critically dependent on the integrity of the NF-κB pathway to survive in adverse conditions, such as those that accompany severe inflammation. For this reason, he suggested efficient blockade of NF-κB activation in a cell-specific fashion in order to inhibit inflammation while preserving hepatocyte survival [44].

In this context, Saavedra *et al* [45] demonstrated the anti-apoptotic effect of NO in the liver using the TNF/D-gal model of massive hepatic apoptosis. Several studies also documented immediate iNOS expression and NO release following partial hepatectomy in rats [46,47]. Hepatocytes appear to be the initial source of NO production, followed by Kupffer and endothelial cells [48,49]. In this setting, transcriptional control of iNOS appears to be regulated by NF-κB. The increased NO level may then be hepatoprotective by reducing apoptosis in hepatocytes. Supporting this argument, iNOS knockout mice subjected to partial hepatectomy demonstrated increased apoptosis 24 h after hepatic resection [49]. In our study, curcumin along with NF-κB (p65) downregulation reduced significantly also iNOS expression in BDL rats which led very probably to decrease NO production associated with fibrotic conditions.

Reyes-Gordillo *et al* [50] has shown, however, beneficial effect of curcumin on macrosurgical model of cholestasis. These authors concluded, that curcumin was effective in preventing and reversing cirrhosis, probably by its ability of reducing TGF-beta expression. The authors suggested that curcumin might be an effective antifibrotic and fibrolitic drug in the treatment of chronic hepatic diseases [50]. Unfortunately, despite a beneficial effect of curcumin on blood plasma markers in BDL rats we did not find decrease of fibrosis extent probably due to concomitant decrease of NF-κB activation discussed above. Similarly, in *in vitro* studies using the rat HSC-T6 cell line curcumin had no direct inhibitory effect on collagen α1 (I) messenger RNA expression. Further studies in these cells using reverse transcriptase-polymerase chain reaction demonstrated that curcumin had no effect on the expression of PDGF-induced TIMP-1 and TIMP-2,

TGFβ1, TGFβ2 and MCP-1 but significantly inhibited tumor necrosis factor alpha expression [32].

In conclusion, we have demonstrated for the first time that in bile duct ligation curcumin treatment was able to improve functional properties of hepatocytes and inhibit both NF-κB and iNOS upregulations, however, without beneficial effect on the liver fibrosis developed in this model of cholestasis. Thus, in our conditions other factors, different from NF-κB and iNOS may be responsible for fibrotic process in the liver.

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Figure Legends

Figure 1: Effect of bile duct ligation (BDL) on blood plasma levels of alkaline phosphatase (ALP) and aspartate aminotransferase (AST) in sham-operated rats (Sham), sham-operated rats administered with curcumin (Curcumin), BDL rats and BDL rats administered with curcumin (BDL curc.). Data are means ± S.E.M., significant differences ($P < 0.05$): * compared to sham operated rats, + compared to BDL.

Figure 2: Effect of bile duct ligation (BDL) on blood plasma levels of total bilirubin (Bil-T) and conjugated bilirubin (Bil-C) in sham-operated rats (Sham), sham-operated rats administered with curcumin (Curcumin), BDL rats and BDL rats administered with curcumin (BDL curc.). Data are means ± S.E.M., significant differences ($P < 0.05$): * compared to sham operated rats, + compared to BDL.

Figure 3: Picrosirius red stained liver sections at low magnification (magn. 100x) in polarized light. Dilated bile ducts with abundant collagen fibers in portobiliar spaces in jaundiced rats. Conspicious increment of collagen fibres in liver parenchyma in curcumin group.

Figure 4: Effect of bile duct ligation (BDL) on liver (FIB-L) and renal (FIB-R) collagen amount in sham-operated rats (Sham), sham-operated rats administered with curcumin (Curcumin), BDL rats and BDL rats administered with curcumin (BDL curc.). Data are

means ± S.E.M., significant differences ($P < 0.05$): * compared to sham operated rats, + compared to BDL.

Figure 5: Effect of bile duct ligation (BDL) on expression of NFκB (p65) and iNOS in sham-operated rats (Sham), sham-operated rats administered with curcumin (Curcumin), BDL rats and BDL rats administered with curcumin (BDL curc.) as percents of sham-operated rats levels. Data are means ± S.E.M., significant differences ($P < 0.05$): * compared to sham operated rats, + compared to BDL.

Figure 6: Immunohistochemical staining of NFκB (p65) in sham-operated rats (Sham), sham-operated rats administered with curcumin (Curcumin), BDL rats and BDL rats administered with curcumin (BDL curcumin). Strong positivity in proliferated biliary ducts in BDL group was partially attenuated by curcumin administration.

Figure 7: Immunohistochemical staining of iNOS in sham-operated rats (Sham), sham-operated rats administered with curcumin (Curcumin), BDL rats and BDL rats administered with curcumin (BDL curcumin). Strong positivity in proliferated biliary ducts in BDL group was partially attenuated by curcumin administration.

Table 1: Effect of curcumin treatment on blood plasma levels of total protein, albumin, creatinine, urea and albumin/globulin ratio in sham operated and BDL rats.

Parameter/Group	Sham	Curcumin	BDL	BDL curc.
Total protein (g/l)	52.45±3.53	49.55±2.06	64.92±7.16*	48.80±1.36^{+}
Albumin (g/l)	33.53±2.09	31.75±1.35	34.04±2.40	26.72±1.72^{+}
A/G ratio	1.77	1.78	1.10*	1.21^{+}
Creatinine (μmol/l)	26.66±3.44	29.16±1.33	26.40±1.43	23.80±2.34
Urea (mmol/l)	7.55±0.51	6.25±0.86	8.92±0.64	6.16±0.31^{+}

Data are means ± S.E.M., significant differences ($P < 0.05$): * compared to sham operated rats, + compared to BDL. A – albumin, G – globulin, BDL – bile duct ligation, curc. – curcumin

Fig. 1

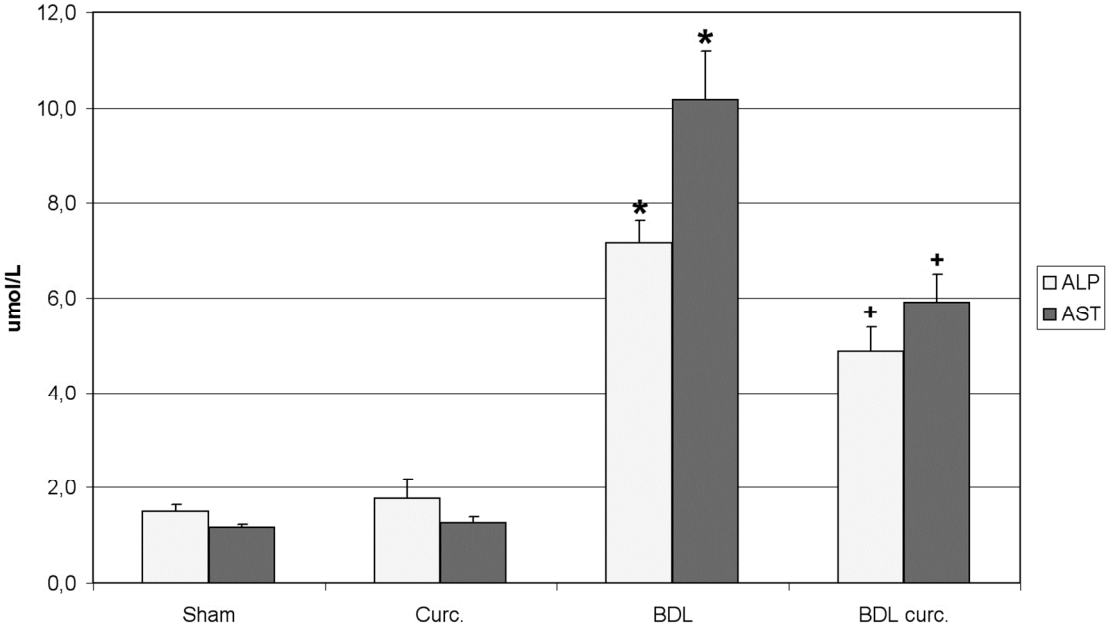

Fig. 2

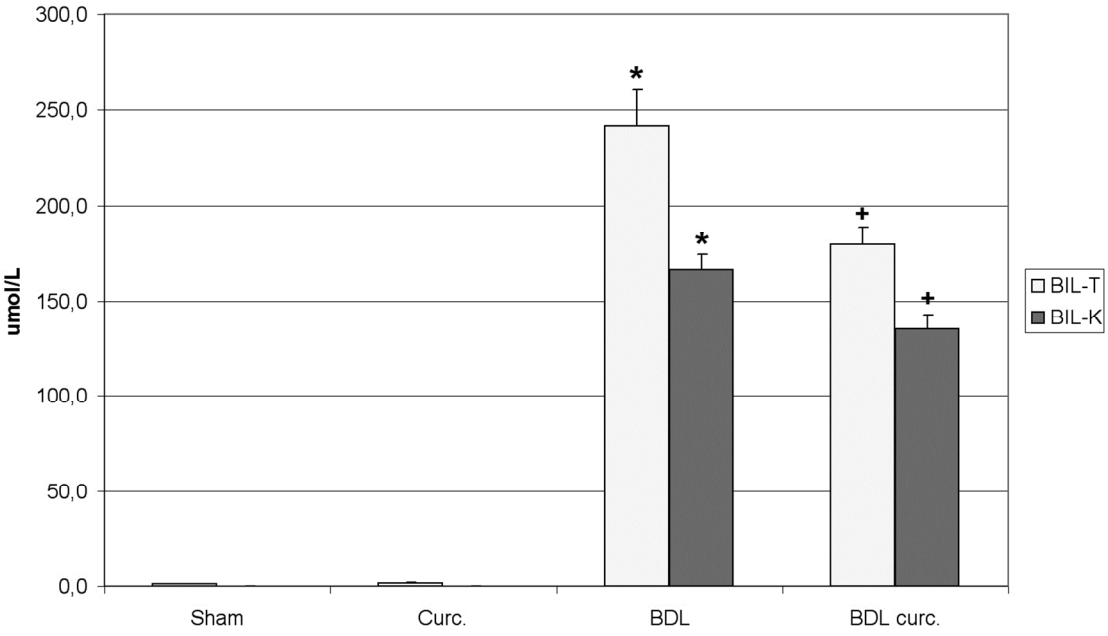

Fig. 3

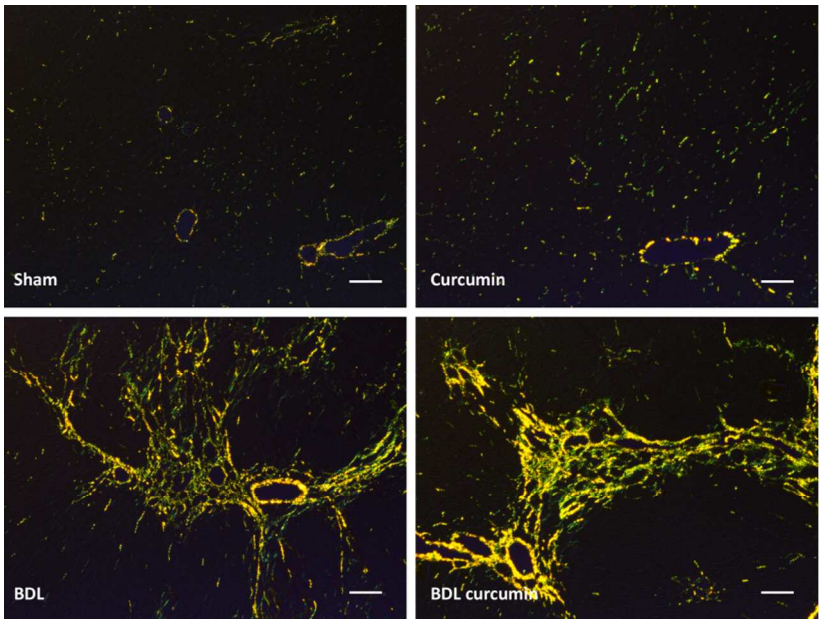

Fig. 4

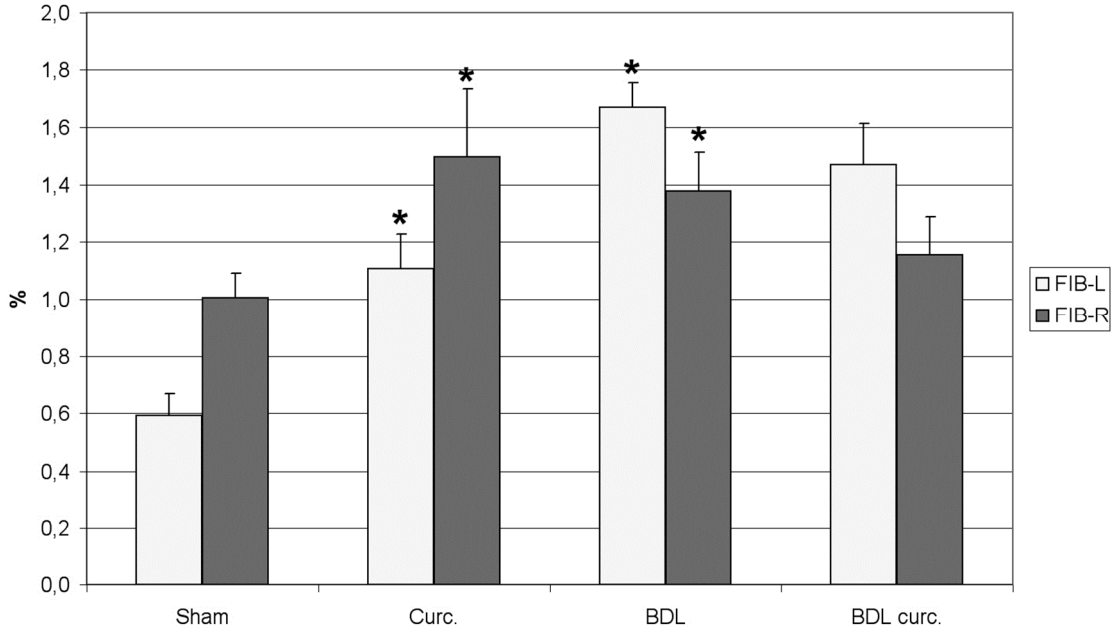

Fig. 5

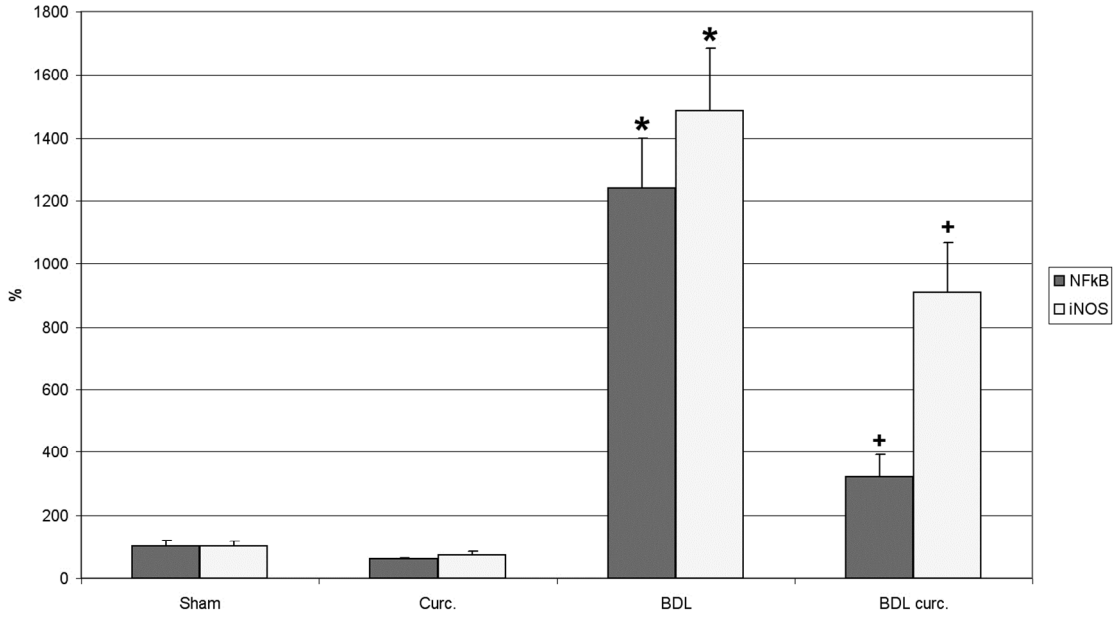

Fig. 6

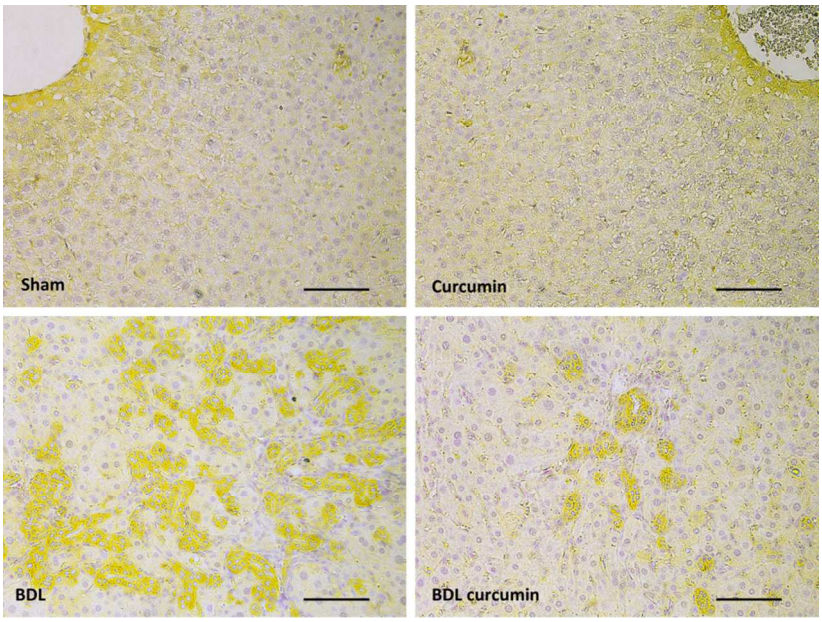

Fig. 7