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Basic Science

AM-1241 CB2 Receptor Agonist Attenuates Inflammation, Apoptosis and Stimulate Progenitor Cells in Bile Duct Ligated Rats

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Keywords: Cannabinoid receptor 2; AM1241; Hepatic progenitor cells; IL-10; p53; Bile duct ligation; Liver fibrosis; CD34

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Abstract

BACKGROUND: The cannabinoid receptor 2 (CB2) plays a pleiotropic role in the innate immunity and is considered a crucial mediator of liver disease. Cannabinoid CB2 receptor activation has been reported to attenuate liver fibrosis in CCl4 exposed mice and also plays a potential role in liver regeneration in a mouse model of I/R and protection against alcohol-induced liver injury.

AIM: In this study, we investigated the impact of CB2 receptors on the antifibrotic and regenerative process associated with cholestatic liver injury.

METHODS: Twenty-six rats had bile duct ligation co-treated with silymarin and AM1241 for 3 consecutive weeks. Serum hepatotoxicity markers were determined, and histopathological evaluation was performed.

RESULTS: Following bile duct ligation (BDL) for 3 weeks, there was increased aminotransferase levels, marked inflammatory infiltration and hepatocyte apoptosis with induced oxidative stress, as reflected by increased lipid peroxidation. Conversely, following treatment with the CB2 agonist, AM-1241, BDL rats displayed a reduction in liver injury and attenuation of fibrosis as reflected by expression of hydroxyproline and α-smooth muscle actin. AM1241 treatment also significantly attenuated lipid peroxidation end-products, p53-dependent apoptosis and also attenuated inflammatory process by stimulating IL-10 production. Moreover, AM1241 treated rats were associated with significant expression of hepatic progenitor/oval cell markers.

CONCLUSION: In conclusion, this study points out that CB2 receptors reduce liver injury and promote liver regeneration via distinct mechanisms including IL-10 dependent inhibition of inflammation, reduction of p53-reliant apoptosis and through stimulation of oval/progenitor cells. These results suggest that CB2 agonists display potent hepatoregenrative properties, in addition to their antifibrogenic effects.

Introduction

Liver fibrosis is a clinical condition, characterised by an accumulation of extracellular matrix proteins as a result of prolonged liver injury. At the same time, there is a continued stimulus for regeneration, prompting further mutilation of the hepatic architecture and vascular structures. If left untreated, fibrosis can progress into cirrhosis, hepatocellular carcinoma and liver failure. The incidence of liver fibrosis has been increased significantly in recent years [1]. Until now, patients suffering from hepatic fibrosis are treated in such a way to compensate such impaired hepatic functions. For quite a long time, liver fibrosis had been considered irreversible.

Nonetheless, there is aggregating clinical and experimental data to propose otherwise. Histological evaluation of biopsies from patients with chronic liver injury and animal models of fibrosis demonstrates that fibrosis is a dynamic, bi-directional process. Thus remodelling of fibrous scar and recovery is conceivable [2].

There is growing evidence suggesting that endocannabinoids may regulate the pathophysiology of liver diseases and plays an important role in apoptosis, tissue homeostasis, cell differentiation and proliferation. Under normal conditions, the endocannabinoid system is quite inactive and CB1, and CB2 receptors are barely expressed, partially because they are not expressed in hepatocytes. However, many studies have demonstrated the up-regulation of the expression of CB1 and CB2 receptors in hepatic myofibroblasts and vascular endothelial cells, as well as the increased concentration of endocannabinoids, especially anandamide, in the liver in the course of chronic

progressive liver diseases [3]. CB1 receptors possess a pro-fibrogenic effect in the liver and have also been implicated in the pathogenesis of alcoholic and nonalcoholic fatty liver diseases [4], [5]. On the other hand, CB2 receptors protect the liver against the development of fibrosis in CCl4 exposed mice [6], [7] and also play a potential role in liver regeneration in mouse model I/R [8] and protection against alcohol-induced liver injury.

Although, CB2 receptor agonists were reported to afford hepatic protection only in a model of I/R and CCl4 [9], [10]. In this study, we have investigated the effects of a CB2 receptor agonist (AM-1241) on a well-established rat model of cholestasis-induced fibrosis induced by three weeks' ligation of bile duct [11], [12], [13]. We have also explored the effects of AM-1241 on the production of the key immune-regulatory cytokine, IL-10 that affect hepatic inflammatory cells and also on oxidative lipid peroxidation and hepatocyte p53- dependent apoptosis. Besides, we have also explored the effects of CB2 receptors on regenerative effect through stimulation of liver progenitor (oval) cells. The latter, in turn, caused further attenuation of liver injury with replacement with healthy hepatocytes. Our findings strengthen the potential of CB2 receptors for the treatment of BDL injury and other fibrotic disorders.

Results were compared to the effects of silymarin, a drug commonly used as liver support during the treatment of cirrhosis. Silymarin has a great activity against a wide range of animal models of hepatic injury due to its antioxidant activity and radical scavenging. Silymarin was also reported to exert anti-inflammatory effects through the reduction of TNF-α [14].

The aim of the study is to evaluate the hepatoprotective effects of the Cannabinoid 2 receptor agonist, AM-1241, on hepatic fibrosis induced by common bile duct ligation of adult rats and to investigate its anti-inflammatory, antioxidant and anti-apoptotic potentials.

Material and Methods

Animals

Twenty-four; Adult male Wistar albino rats, of approximately 180-220 g body weight, were obtained from the animal house colony, National Research Centre, Giza, Egypt. All animals were housed in metal cages in a well-ventilated environment at (22 ± 3°C, 55 ± 5% humidity and 12h dark & light cycles) and were provided with a standard pellet diet (containing not less than 20% protein, 5% fiber, 3.5% fat, 6.5% ash and a vitamin mixture) and water ad libitum. All animal care and experimental procedures were approved by Ethical Committee of National research centre, Egypt (registration number: 16-036) and followed the ARRIVE (Animal Research: Reporting In-Vivo Experiments) guidelines [15].

Material

AM1241 was purchased from CaymanChem, Czech Republic. Silymarin was granted from CID, Egypt. Diethyl ether, Formaldehyde solution 34-38%, DMSO and PBS were obtained from Sigma, Egypt. Ketamine, Thylacine and Ceftriaxone have obtained from Egyptian companies; SIGMA TEC, ADWIA and EPICO respectively.

General procedures

Bile duct ligation (BDL) has been widely used for experimental induction of liver fibrosis in rats [16]. For this purpose, rats were anaesthetized with ketamine (50 mg/kg, i.p) and thylacine (5 mg/kg, i.p) [17] then the abdomen was shaved and disinfected. The common bile duct was exposed and twice ligated with 3-0 silk suture. Sham operation was performed by gently touching the bile duct without ligation. The abdomen was closed in layers. The animals were allowed to recover on a heating pad. Rats were injected with ceftriaxone (30 mg/kg, im) 30 minutes before surgery for prophylaxis against infection [18]. The experimenters were blinded to the treatments given to the animals and the biochemical and histological analyses and the data analyses.

Experimental design

The day after the operation, animals were randomly divided into four groups of six rats each and treated for three consecutive weeks as follows: The first group was sham-operated and served as the non-treated control, receiving vehicle only. The second group had a bile duct ligation then it was given the vehicle (BPS/DMSO solution in a ratio of 2:1), intraperitoneally. The third group also had bile duct ligation then was given silymarin daily in a dose of 100 mg/kg dissolved in the vehicle, intraperitoneally [19]. The fourth group as well had bile duct ligation then was given AM1241 daily in a dose of 3 mg/kg dissolved in the vehicle, intraperitoneally [20]. After three weeks (at 24 h after the last injection), blood samples were collected from the tail vein, and serum was separated by centrifugation at 3000 × g for 10 min and was used for the biochemical assessment. Rats were sacrificed by cervical dislocation, and livers were dissected, weighed, and liver to body weight ratio was calculated.

Serum biochemistry of liver transaminases, ALP and bilirubin levels

Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total and direct bilirubin were determined using colourimetric kits (Biodiagnostic, Cairo, Egypt) [21], [22], [23].

ELISA for hepatic MDA, GSH, TNF-α, IL-10 and hydroxyproline content

Liver tissues were washed and homogenised in ice-cold PBS (pH = 7.4) to obtain a 20% homogenate (w·v 1), which was then centrifuged for 15 min at 3000 x g and 4°C. The supernatant obtained was used for measuring MDA (Bioassay co., China), GSH (Biotech co., China), TNF-α (Cusabio co., China), IL-10 (Cusabio co., China) and hydroxyproline (Cusabio co., China) using sandwich ELISA kits according to the manufacturer's instructions. Results were expressed for GSH, TNF and IL-10 as pg/mg protein while for MDA were expressed as nmol/mg and for hydroxyproline as ng/g [24], [25], [26], [27].

qRT-PCR analysis of α-FP and CD34 gene expression

Total RNA was isolated from rat liver samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription and quantitative real-time PCR (qRT-PCR) were performed using SYBR® Premix

Ex TaqTM (TaKaRa, Biotech. Co. Ltd.). The cycling conditions were as follows: reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The relative mRNA level of the target genes was calculated by the comparative threshold cycle (Ct) method and was normalised on the bases of β-actin expression. The fold change in the expression of each target gene was calculated by the following formula: relative quantification = 2ΔΔCt. The following primer sequences were used: CD34 forward primer: 5′- AGC CCT ACA GGA GAA AGG CTG -3′, CD34 reverse primer: 5′- TCA CAG TTC TGT GTC AGC CAC -3′, β-actin forward primer: 5′- TTT GCA GCT CCT TCG TTG CC-3′, β-actin reverse primer: 5′- CGG TTG GCC TTA GGG TTC AGG GGG G-3′, α-FP forward primer: 5′- AGC GAG GAG AAA TGG TCC GG -3′, α-FP reverse primer: 5′- GGA CAT CTT CAC CAT GTG G -3′ (Metabion, Germany) [28], [29].

Histopathological examination

Autopsy samples were taken from the liver of rats in different groups and fixed in 10% formol saline for twenty-four hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degrees in a hot air oven for twenty-four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns' thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin &eosin stain as well as Masson Trichrome for routine examination through the electric light microscope [30]. The severity of histopathological alternation was semi-quantitatively assessed based on liver histology evaluated by a blinded pathologist using a scoring system in which score 0 indicated no alternation; score 1, mild alternation; score 2, alternation activity; score 3, severe alternation.

Immunohistochemical analysis of α-SMA, p53, α-FP and CD34

Paraffin-embedded liver sections were deparaffinized and hydrated. Immunohistochemical analyses were performed by a standard streptavidin-biotin-peroxidase procedure. The paraffin sections were heated in a microwave oven (25 min at 720 W) for antigen retrieval and incubated with one of the following primary antibodies: rabbit polyclonal anti-rat p53, rabbit polyclonal anti-rat a-FP, rabbit monoclonal anti-rat CD34 and rabbit monoclonal anti-rat α-SMA (1:50 dilution; Abcam, Cambridge, MA, USA) and incubated overnight at 4°C. After washing with PBS, followed by incubation with the corresponding biotinylated secondary antibody (1:200 dilutions; Dako Corp.) and streptavidin/alkaline phosphatase complex (1:200 dilutions; Dako Corp.) for 30 min at room temperature, the binding sites of antibody were visualised with DAB (Sigma). After washing with PBS, the samples were counterstained with H&E for 2–3 min, and dehydrated by transferring them through increasing ethanol solutions (30%, 50%, 70%, 80%, 95%, and 100% ethanol). Following dehydration, the slices were soaked twice in xylene at room temperature for 5 min, mounted, examined, and evaluated by a high-power light microscope [31].

Quantitative morphometric analysis of Masson's trichrome, α-SMA, p53, α-FP and CD34

Quantitative morphometric analysis for Masson's trichrome, α-SMA, P53, α-FP and CD34 was performed at the Pathology Department, National Research Center by measuring the percentage of the positive stained area using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England). Morphometric measurements were performed on real-time image from the microscope that was visualised on the video monitor at high power magnification (200 ×). The marker colour to be detected was selected, then the software formed a binary image for the area of stained by the marker.

This area is determined as an area per field in micrometre square, area fraction and area percentage by using the interactive measurement software of the system. The results appeared in the form of mean, standard deviation, standard error, the minimum area and the maximum area measured. Quantitative image analysis for the selected marker was expressed as the percentage of stained area averaged across 5 different fields for each rat of at least six rats [32].

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on the experimental analysis in pharmacology [33]. Results are expressed as mean ± SE. Multiple comparisons were performed using two-way ANOVA followed by Tukey-Kramer as a post hoc test. $P < 0.05$ was considered statistically significant. All analyses and graphs were performed using GraphPad Prism software (version 7).

Results

AM1241 treatment amended BDL-induced hepatic damage

As compared with the sham group, ALT, AST and ALP serum levels were significantly elevated in the BDL group. On the other hand, the levels of ALT, AST and ALP were significantly less elevated with AM1241 treated rats by 21%, 31% and 29% respectively, compared to that of the BDL group levels but they remained significantly higher than the sham values. In line with the cholestatic injury, the BDL group also showed a significant rise in total and direct bilirubin, compared to the control as they are not excreted through the obstructed bile duct and due to damaged hepatocytes. However, these values were significantly less elevated in AM1241 treated rats by 19%, 27% respectively than nontreated BDL group; moreover, it remained significantly higher than the silymarin group.

Table 1: Effect of AM1241 (3 mg/kg b.wt.) and silymarin (50 mg/kg b.wt.) treatment for three weeks on hepatotoxicity indices, liver and body weight ratio in bile duct ligated (BDL) rats

Groups	ALT	AST	ALP	Total Bilirubin	Direct Bilirubin	Change in body weight	Liver to body weight ratio
Sham	22.83 ± 0.61^{bc}	24.33 ± 0.92^{bc}	41.67 ± 0.71^{bc}	0.61 ± 0.06^{bc}	0.17 ± 0.014^{bc}	32.5 ± 8.04^{bc}	0.031 ± 0.001^{bc}
BDL	82 ± 2.35^{ac}	114 ± 2.89^{ac}	132.33 ± 2.47^{ac}	21.81± 0.90^{ac}	10.58 ± 0.54^{ac}	-23 ± 9.61^{a}	0.065 ± 0.002^{ac}
BDL + Silymarin	56 ± 1.34^{ab}	66.33 ± 2.33^{ab}	82.67 ± 2.23^{ab}	12.23 ± 1.6^{ab}	6.5 ± 0.44^{ab}	-17.27 ± 14.02^{a}	0.056 ± 0.002^{ab}
BDL + AM1241	64.83 ± 2.91^{abc}	78.87 ± 2.29^{abc}	94 ± 1.63^{abc}	17.63 ± 0.56^{abc}	7.7 ± 0.51^{ab}	-17.5 ± 10.01^{a}	0.051 ± 0.002^{ab}

Bile duct ligated rats significantly lost weight as compared to sham rats, moreover AM1241 and silymarin treated rats also lost weight without a difference with BDL group. Regarding liver to weight ratio, the increase observed in the BDL group, compared to the sham group, was significantly reduced by the administration of AM1241 and silymarin (Table 1).

Moreover, liver from bile duct ligated rat liver showed a nodular yellow brownish surface with oedema formation, distended bile cyst and fatty changes. However, treatment with AM1241 and silymarin produced a smooth, faint brownish surface (Figure 1).

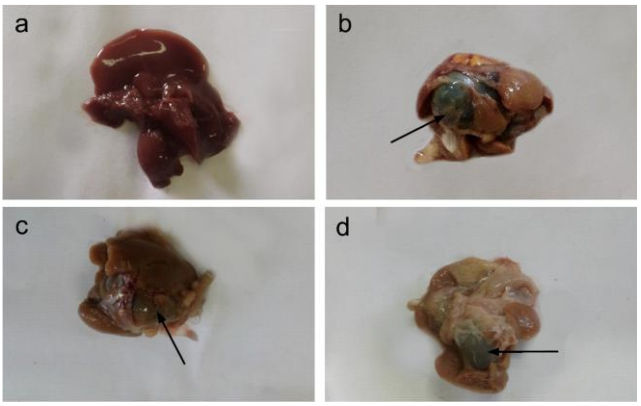

Figure 1: Effect of AM1241 (3 mg/kg) and silymarin (100 mg/kg) treatment for three weeks on gross liver morphology in bile duct ligated (BDL) rats. Liver from sham rat shows normal brownish surface (A), bile duct-ligated rat liver shows a nodular yellow brownish surface with oedema formation, distended bile cyst (arrow) and fatty changes (B), while liver treated with silymarin shows a smooth faint brownish surface with distended bile cyst (arrow) (C). Moreover, AM1241 treated rats show similar smooth, faint brownish surface (D)

AM1241 treatment inhibited histopathological deterioration induced by BDL

H&E stained liver sections obtained from sham group revealed no histopathological alteration and the normal histological structure of the central vein and the portal area with the portal vein, hepatic artery and bile ducts were recorded in (Figure 2a and 2b). However, in bile duct ligated rats, coagulative necrosis was observed in some hepatocytes which were characterised by deep eosinophilic cytoplasm and pyknotic deep blue nuclei in association with another dysplastic one with karyocytomegaly (Figure 2c, 2d and 2e). The hepatic capsule (Glisson's Capsule) showed thickening associated with a fatty change in the underlying hepatocytes (Figure 2f and 2g). Massive inflammatory cells infiltration was detected in the portal area associated with hyperplasia with the atypical epithelium of the bile ducts extended to the hepatic parenchyma (Figure 2h and 2i). There was neocholangiolar proliferation of the bile ducts characterised by hyperplasia and hypertrophy with ductular and glandular structure presented in between the damage hepatocytes as regenerative effects (Figure 2j).

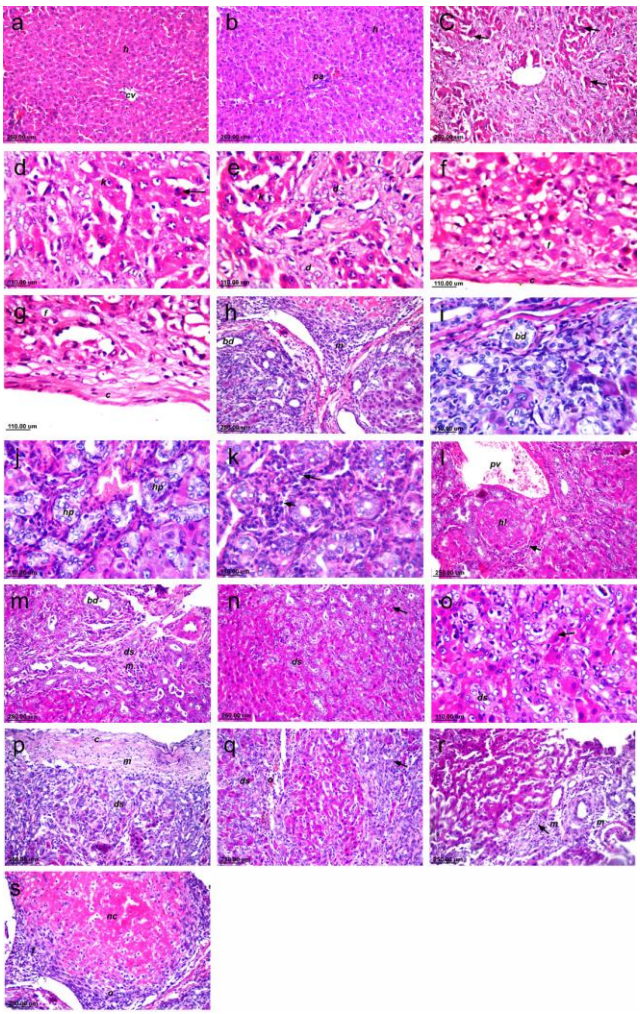

Figure 2: AM1241 treatment (3 mg/kg for 3 weeks) alleviated histopathological deterioration using H&E staining (×40 & ×80) in hepatic fibrosis associated with bile duct ligation (BDL). Liver sections of (a & b) sham rats showing the normal histological structure of the central vein (cv), surrounding hepatocytes (h) in the parenchyma, and normal portal area (pa). (c, d & e) BDL liver tissue is showing coagulative necrosis (arrow) with deeply eosinophilic cytoplasm and pyknotic nuclei hepatocytes while other dysplastic (d) with disfiguration and cytomegaly and karyomegalocytic cells (k). (f & g) BDL liver tissue is showing thick capsule (c) with fatty changes (f) in underlying hepatocytes. (h & i) BDL liver tissue is showing massive inflammatory cell infiltration (m) in the portal area with hyperplastic bile duct cells resembling hepatocytes, biliary epithelium and extending to the parenchyma (bd). (j) BDL liver tissue is showing hyperplasia and hypertrophy (hp) with ductular glandular structure presented in freshly damage liver cells as a regenerative effect (neocholangiolar proliferation). (k) BDL liver tissue is showing oval or spindle cell (arrow) basophilic extended in between the hyperplastic hypertrophic bile ducts in the hepatic parenchyma. (l) silymarin treated liver tissue showing dilated portal vein (pv) with lobulation of the hepatocytes (hl) with multiple proliferated oval or spindle cells (arrow) and dysplasia (ds) in the other with prominent nucleoli. (m) silymarin treated liver tissue showing increase proliferation of the bile ducts (bd), hypertrophic biliary epithelium, atypia and inflammatory cell infiltrate (m) in portal area. (n & o) silymarin treated liver tissue showing coagulative necrosis in some of the hepatocytes, dysplasia in other with enlarged spindle cells extended in between in the parenchyma. (p) AM1241 treated liver tissue showing thickening (c) with inflammatory cells infiltration (m) in the hepatic capsule with dysplasia (ds) in the underlying hepatocytes and atrophy in other. (q) AM1241 treated liver tissue showing oval cells proliferation (o) in between the dysplastic hepatocytes (arrow). (r) AM1241 treated liver tissue showing inflammatory cells infiltration (m) with few fibroblastic cells proliferation in the portal area (arrow). (s) AM1241 treated liver tissue showing focal loss of cell detail and architecture as necrosis (ne) surrounded by fibrosis (f) and oval cells (o)

Oval or basophilic spindle cells were extended in between the hyperplastic hypertrophied ducts in the parenchyma (Figure 2k). In silymarin treated rats, hepatic capsule showed thickening with inflammatory cells infiltration while the underlying parenchyma had dysplasia and atrophy (Fig. 2p). There was oval cells proliferation in between the dysplastic hepatocytes (Figure 2q). Inflammatory cells infiltration with few fibroblastic cells proliferation was detected in the portal area (Figure 3r). Focal necrosis with fibrosis in the surrounding was detected in the hepatic parenchyma (Figure 2s). Rat treated with AM1241 showed dilatation was observed in the portal vein associated with the proliferation of the oval or spindles cells which were separated the dysplastic with prominent nucleoli hepatocytes into lobules (Figure 2l). There was an increase in the bile ducts proliferation with hypertrophic biliary epithelium, atypia and inflammatory cells infiltration (Figure 2m). Coagulative necrosis was detected in some of the hepatocytes associated with dysplasia in others as well as an extension of spindle cells proliferation in between (Figure 2n & 2o). Table 2 summarise the histopathological alterations demonstrated in liver tissue of BDL rats.

Table 2: Effect of administration of AM1241 (3 mg/kg b.wt.) and silymarin (50 mg/kg b.wt.) for three weeks on histopathological alternations of hepatic tissue in bile duct ligated (BDL) rats

Groups	Hepatic capsule thickening	Degeneration and necrosis of hepatic parenchyma	Dysplasia of hepatocyte	Hyperplasia, hypertrophy and neocholangiolar formation with atypia, cytomegaly	Portal inflammatory reaction	Oval cell proliferation	Fibrosis
Sham	0	0	0	0	0	0	0
BDL	1	3	3	3	3	2	2
BDL + Silymarin	2	2	2	2	2	2	2
BDL + AM1241	0	2	2	2	1	3	1

AM1241 treatment mitigated BDL-induced hepatic fibrogenesis

ELISA analysis showed that that bile duct ligation caused a significant rise in hepatic hydroxyproline levels (up to 3 folds), compared to that of the control group. However, silymarin group didn't show significant difference with BDL rats, AM1241 treatment successfully reduced the elevation of hydroxyproline levels, compared to that of the BDL group (Table 3).

Table 3: AM1241 treatment (3 mg/kg for 3 weeks) alleviated hepatic fibrogenesis in bile duct ligated (BDL) rats

Groups	HP	MT %	SMA %
Sham	1.03 ± 0.06^{bc}	17.12 ± 1.88^{bc}	7.11 ± 1.29^{bc}
BDL	4.09 ± 0.09^{a}	39.76 ± 2.10^{a}	39.03 ± 2.30^{ac}
BDL + Silymarin	4.02 ± 0.20^{a}	32.61 ± 2.12^{a}	16.55 ± 1.98^{ab}
BDL + AM1241	3.29 ± 0.31^{ab}	30.04 ± 2.25^{ab}	14.30 ± 1.29^{ab}

These results matched those obtained from histomorphometric measurements of Masson Trichrome staining. It was clear that only traces of connective tissue elements were present in normal

hepatic tissues (Figure 3a), while bile duct ligation increased markedly the fibrous components associated with portal fibrosis (Figure 3b). Treatment of bile duct ligated rats with silymarin produced a less significant reduction of fibrosis than that of AM1241 group (Figure 3c and 3d). However, treatment with AM1241 significantly decreased fibrosis by 24% compared to the nontreated BDL group (Figure 3e, 3f and 3g).

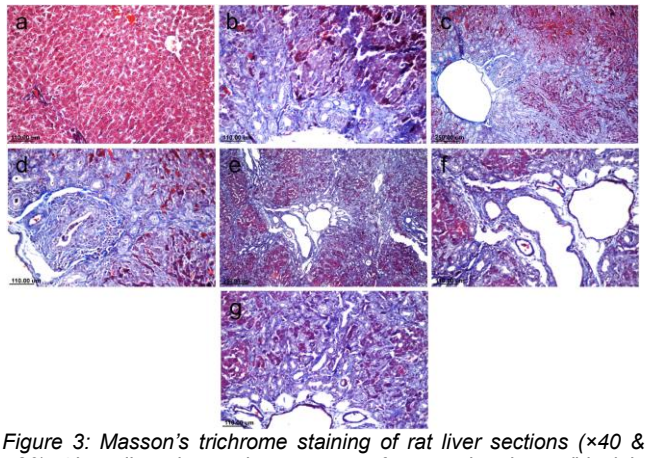

Figure 3: Masson's trichrome staining of rat liver sections (×40 & ×80). Sham liver tissue shows traces of connective tissue (blue) in the normal hepatic tissue present mainly around the main blood vessels (a). Bile duct-ligated rat liver tissue shows massive fibrosis in liver tissue with thick fibrous tissue expanded along the portal tract and extended into the periportal region. Lost normal architecture and dilated bile ducts was also observed (b). Moreover, the liver treated with silymarin shows also marked decrease in connective tissue (c & d). However, liver treated with AM1241 shows only a few blue stained collagen bundles surrounding hepatic nodules (e, f & g)

Moreover, quantitative morphometric investigation of liver sections immunostained with α-SMA, a marker of fibroblastic cells, i.e. activated hepatic stellate cells and portal myofibroblasts revealed that only traces of α-SMA expression was present in normal hepatic tissues (Figure 4a). In contrast, bile duct ligation produced the highest expression of α-SMA in hepatic tissue (Figure 4b and 4c). Treatment with silymarin produced a similar insignificant effect to that of AM1241 treated rats (Figure 4d). However, treatment with AM1241 significantly decreased α-SMA expression by 63%, compared to that of the nontreated BDL group (Figure 4e and 4f).

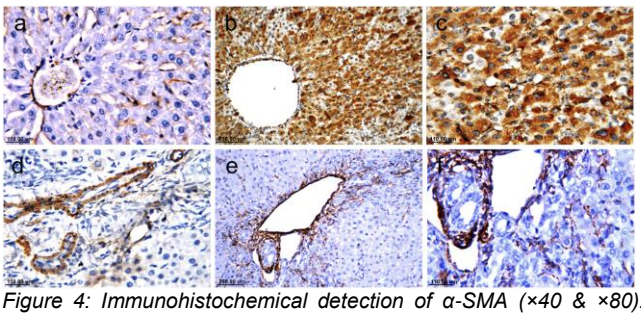

Figure 4: Immunohistochemical detection of α-SMA (×40 & ×80). Sham group shows a negligible positive reaction (a), bile duct ligated group shows highest reaction (b & c), group treated with silymarin shows decrease in reaction (d), while AM1241 treated group shows a more noticeable decrease in reaction (e & f)

AM1241 treatment attenuated BDL-induced oxidative stress

The effect of AM1241 on oxidative stress was investigated by measuring hepatic levels of the lipid peroxidation end-product (MDA; malondialdehyde) and the antioxidant molecule (GSH). GSH levels were markedly reduced while levels of lipid peroxides were significantly elevated in the BDL group, compared to that of the control group. Interestingly, Treatment with AM1241 significantly prevented the elevation of MDA by 45% and restored GSH levels up to 5 more folds than the nontreated BDL group (Table 4). Moreover, AM1241 exhibited a superior effect when compared to that of silymarin treated group.

Table 4: Effect of administration of AM1241 (3 mg/kg b. wt.) and silymarin (50 mg/kg b. wt.) for three weeks on tissue oxidative stress markers in bile duct ligated (BDL) rats

Groups	MDA (nmol/mg)	GSH (pg/mg)
Sham	31.67 ± 1.09^{bc}	122.67 ± 2.45^{bc}
BDL	182.167 ± 6.41^{ac}	14.87 ± 0.89^{ac}
BDL + Silymarin	134.67 ± 3.99^{ab}	70.57 ± 3.94^{ab}
BDL + AM1241	99 ± 3.02^{abc}	89.8 ± 2.89^{abc}

AM1241 treatment alleviated BDL-induced hepatic inflammation

The anti-inflammatory potential of AM1241 was determined by measuring levels of the pro-inflammatory cytokine (TNF-α) and the anti-inflammatory marker (IL-10). As shown in Table 3, the marked rise in serum TNF-α levels observed in the BDL group was significantly decreased (33%) by AM1241 treatment. Silymarin produced a more significant decrease in TNF-α levels, compared to that of AM1241 group.

Regarding IL-10 serum levels, bile duct ligated rats showed a significant decrease, compared to the control group, whereas treatment with AM1241 markedly restored these levels up to 5.6 more folds than the nontreated BDL group. Further, treatment with silymarin showed a significant inferior effect when compared to AM1241 group (Table 5).

Table 5: Effect of administration of AM1241 (3 mg/kg b.wt.) and silymarin (50 mg/kg b.wt.) for three weeks on tissue inflammatory response in bile duct ligated (BDL) rats

Groups	TNF-α (pg/mg)	IL-10 (pg/mg)
Sham	37.27 ± 1.28^{bc}	124.37 ± 1.78^{bc}
BDL	223.53 ± 3.75^{ac}	14.43 ± 0.85^{ac}
BDL + Silymarin	130.8 ± 6.15^{ab}	66.2 ± 3.82^{ab}
BDL + AM1241	149.2 ± 4.48^{abc}	95.83 ± 2.18^{abc}

AM1241 treatment alleviated p53-dependent hepatocyte apoptosis

Table 6 summarizes the quantitative morphometric investigation of liver sections immunostained with p53 of BDL rats. Liver sections of normal hepatic tissues showed a negligible positive

reaction of p53 expression (Figure 5a). Bile duct ligation produced a large increase in p53 immunoreactivity (Figure 5b and 5c).

Table 6: AM1241 treatment (3 mg/kg for 3 weeks) alleviated hepatic fibrogenesis in bile duct ligated (BDL) rats

Groups	P53
Sham	5.53 ± 0.53[bc]
BDL	32.77 ± 2.44[ac]
BDL + Silymarin	23.85 ± 1.84[ab]
BDL + AM1241	15.89 ± 1.89[abc]

Silymarin treatment was less effective in reducing p53 induction (Figure 5d), and treatment with AM1241 significantly decreased p53 expression significantly by 51%, compared to that of the nontreated BDL group (Figure 5e).

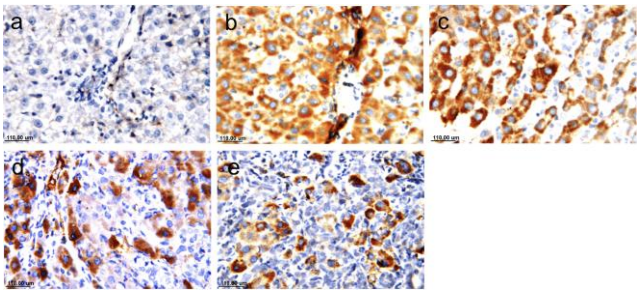

Figure 5: Immunohistochemical detection of p53 (×40 & ×80). Sham group shows a negligible positive reaction (a), bile duct ligated group shows highest reaction (b & c), group treated with silymarin shows decrease in reaction (d), while AM1241 treated group shows a more noticeable decrease in reaction (e)

AM1241 treatment augmented liver regeneration by activating hepatic progenitor cell proliferation

The effect of AM1241 on liver regeneration through activation of hepatic progenitor cells proliferation was determined by measuring levels of its surface markers, CD34 and α-FP. qRT-PCR analysis of CD34 and α-FP mRNA expression in the bile duct ligated group showed no significant changes from the control group. Silymarin treatment didn't show any significant effect over the nontreated BDL rats. Strikingly, treatment with AM1241 significantly up-regulated the genetic expression of CD34 and α-FP (252% and 89% respectively), compared to that of the nontreated BDL group (Table 7).

Table 7: AM1241 treatment (3 mg/kg for 3 weeks) augmented hepatic progenitor cell expression in bile duct ligated (BDL) rats

Groups	AFP %	AFP PCR	CD34 %	CD34 PCR
Sham	14.904 ± 3.32[bc]	0.39 ± 0.04[bc]	3.70 ± 0.81[bc]	1.14 ± 0.11
BDL	34.30 ± 3.09[a]	0.83 ± 0.03[a]	17.55 ± 2.15[a]	2.32 ± 0.43
BDL + Silymarin	41.69 ± 2.50[a]	0.755 ± 0.036[a]	23.59 ± 2.66[a]	2.20 ± 0.49
BDL + AM1241	55.03 ± 2.23[abc]	1.57 ± 0.07[abc]	38.18 ± 1.91[abc]	8.17 ± 1.021[abc]

Histomorphometric measurement for CD34 and α-FP immunostained liver sections demonstrated a very similar pattern as the qRT-PCR measurements. Only traces of CD34 and α-FP were found in normal hepatic tissues (Figure 6a, 6b and 7a). Bile duct ligated treated rats showed mild expression of CD34 (Figure 6c, 6d, 7b and 7c). Moreover, silymarin treated rats showed also a mild expression of when stained with CD34 (Figure 7d and 7e).

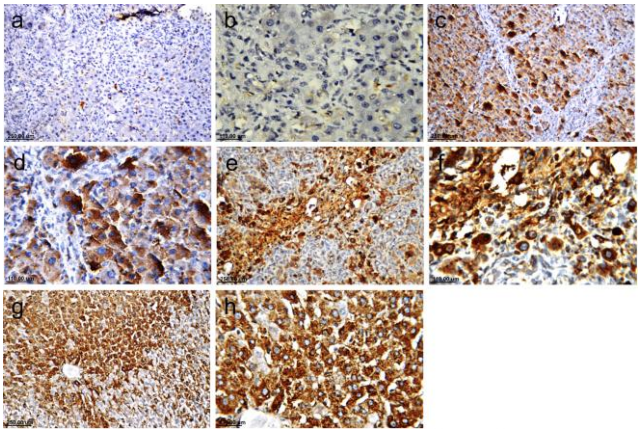

Figure 6: Immunohistochemical detection of α-FP (×40 & ×80). Sham group shows the negligible positive reaction of this stain (a & b), a noticeable increase in the reaction in BDL group (c & d) and silymarin treated group (e & f) and highest reaction in AM1241 treated group (g & h)

However, it revealed more significant expression when stained with α-FP (Figure 6e and 6f). Treatment with AM1241 showed the highest expression of CD34 and α-FP (117% and 60% respectively, compared to BDL group) with observed oval hepatic progenitor cells (Figure 6g, 6h, 7f, 7g).

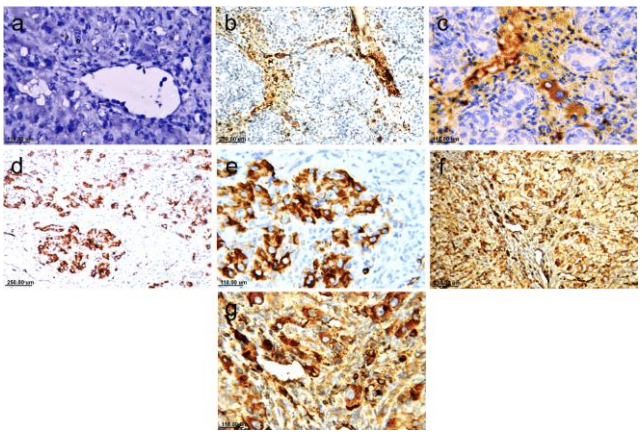

Figure 7: Immunohistochemical detection of CD34 (×40 & ×80). Sham group shows no positive reaction (a), mild increase in the reaction in both BDL (b & c) and silymarin groups treated groups (d & e) and highest reaction in the group treated with AM1241 (f & g). Notice the hepatic progenitor cells is well expressed in AM1241 treated group

Discussion

The current study reports the potential antifibrotic mechanisms of CB2 receptors activation through studying the key events involved in hepatic

fibrosis such as oxidative stress, inflammation and apoptosis. This study is the first to investigate the potential effect of CB2 receptor on liver regeneration associated with hepatic progenitor cells stimulation. These effects largely depend on its direct impact on CB2-expressing hepatic immune cells and hepatic myofibroblasts and its indirect effect on hepatocytes, which do not express CB2 [8], [34].

BDL is the classic experimental model for induction of cholestatic liver fibrosis in rodents. In our study following 3 weeks of bile duct ligation, there was a marked decrease in body weight associated with an increase in liver weight. Reduced body weight could be attributed to reduced intestinal bile secretion and food absorption while increased liver weight is referred to as hepatic congestion and swelling [35]. AM1241 treatment significantly attenuated body weight loss and liver weight increase.

Biliary obstruction causes an irregular flux of bilirubin and bile acids in the liver. Toxic hydrophobic bile salts subsequently accumulate within hepatocytes causing progressive hepatocellular necrosis and apoptosis via free radical generation. Early studies using rodent hepatocytes assumed that cholestatic liver injury is mainly caused by direct toxicity of hydrophobic bile acids, which induce mitochondrial oxidative stress and apoptosis [36]. However, the predominant bile acids in rodents are not cytotoxic; they promote inflammatory gene expression which is responsible for hepatic neutrophil recruitment and an inflammatory injury. Neutrophil cytotoxicity involves extensive neutrophil-derived oxidative stress and hepatocyte necrosis [37]. This was reflected in the present study by the elevation in serum transaminases, ALP and bilirubin in the BDL group.

Moreover, Histopathological examination revealed coagulative necrosis in association with many dysplastic cells and hepatocytes showing fatty changes. Massive inflammatory cells infiltration was detected in the portal area associated with the neo-cholangiolar proliferation of the bile ducts as regenerative effects. Oval basophilic cells were extended in between the hyperplastic ducts. Daily administration of AM1241 produced significant decrease in serum transaminases, ALP and bilirubin levels with no significant difference from silymarin group. However, histopathological examination still reveals areas of coagulative necrosis in some of the hepatocytes associated with few dysplastic cells as well as proliferation of oval cells in between. Moderate Inflammatory cells infiltration with few fibroblastic cells proliferation was detected in the portal area**.** These results confirm the previous findings by Batkai, Osei-Hyiaman [9] who reported that administration of CB2 agonist, JWH-133 protected against hepatic I/R (ischemia reperfusion) injury whereas, CB2 deficient mice displayed enhanced liver injury and inflammation following I/R. The reported mechanism involved decreased inflammatory cell infiltration, reduced lipid peroxidation and expression of pro-inflammatory cytokines. Moreover, ***Batkai, Osei-Hyiaman [9]*** experiments in cultured sinusoidal endothelial cells indicated that CB2 activation reduced tumor necrosis factor alpha-induced adhesion molecules with decreased adhesion of neutrophils to endothelial cells.

Liver fibrosis of any aetiology is characterized by progressive accumulation of collagenous fibrous tissue in the liver parenchyma with the proliferation of collagenous secreting activated hepatic stellate cells (HSCs) and portal myofibroblasts [38]. In this context, BDL in our study produced marked collagenous filaments deposition around portal tract as indicated by Masson's trichrome stained fibrous bands, increased liver hydroxyproline content (a marker of collagen deposition) and also by increased α-SMA expression (a marker of HSCs). The present study demonstrates that CB2 activation successfully decreased BDL-induced fibrogenesis. This was concluded by the significant reduction in liver hydroxyproline levels, measured with ELISA and the decrease of collagen deposition, measured with morphometric analysis of Masson's stain. This also was coincided with a significant reduction in α-SMA expression. These results confirm the previous findings by Julien, Grenard [6] who reported that CB2 knockout mice showed a diminished cirrhosis when exposed to CCl4, as assessed by morphometric analysis of Sirius red-stained slides, and by measurement of collagenous protein spectrophotometrically, whereas Munoz-Luque, Ros [39] reported that administration of the CB2 agonist JWH-133 to CCl4 rats improved liver fibrosis, decreases the inflammatory infiltrate and reduces the density of hepatic myofibroblasts.

Moreover, Julien et al**.,** 2005 also reported that activation of CB2 receptors in cultured activated hepatic stellate cells reduced cell accumulation and triggered its apoptosis by stimulating both COX-2 and oxidative stress. They reported that THC increased COX-2 protein expression and ROS activity that induced HSCs apoptosis. However, the apoptosis was diminished by selective COX-2 inhibitor and two potent antioxidants, respectively.

CB2 receptors expressed on hepatic inflammatory cells play a major role in amelioration of hepatic injury and fibrogenesis. Previous literature showed that CB2 agonists prevented the switch of Kupffer cells to a pro-inflammatory M1 phenotype, and enhances transition towards the anti-inflammatory M2 phenotype, via a mechanism involving activation of heme oxygenase-1 [40]. While others reported that CB2 receptors activation downregulated the production of the profibrogenic cytokine IL17 by Th17 lymphocytes [41], [42]. Moreover, our finding showed that CB2 receptors produced a marked elevation of immunoregulatory IL-10 as compared to both BDL and silymarin groups. IL-10 produced by M2b macrophages and Th2 lymphocytes is a potent stimulator of HO-1 with subsequent enhancing polarisation of macrophages toward

immunoregulatory M2c phenotype [43], [44], [45]. Also, IL-10 produces apoptosis of pathogenic Th17 and decreases the release of inflammatory IL-17 [46], [47], [48]. This was evident in our experiment by a significant decrease of TNF-a, a marker of pro-inflammatory M1-macrophages and by a significant increase of IL-10, a marker of anti-inflammatory M2-macrophages.

Neutrophil accumulation in the liver is a common feature of the cholestatic liver disease and is mediated by several adhesion molecules and inflammatory cytokines released from kupffer cells. These inflammatory cells attach to hepatocytes and release ROS and other oxidants. These changes, in turn, trigger intracellular oxidative stress and lipid peroxidation inside the hepatocytes [49], [50]. This was evident in the present study by the remarkable increase in lipid peroxidation expressed as MDA content and the depletion of GSH levels. Oxidative stress is found to play a major role in the pathogenesis of cholestasis as it induces hepatocyte death (both apoptotic and necrotic), amplifies the inflammatory response. Treatment of BDL rats with CB2 agonist in the present study effectively reduced the lipid peroxidation and restored the hepatic antioxidant defence system as shown by the marked improvement of MDA and GSH activity which was coincided with a decreased number of inflammatory cells as shown by H&E stain, compared to both BDL and silymarin groups. This goes in line with previous studies which reported that activation of CB2 receptors by JWH133 and cannabidiol decreased hepatic ROS and lipid peroxidation end-products by attenuating neutrophil infiltration and expression of neutrophil adhesion molecules in an animal model of I/R and alcohol-induced injury, respectively [9], [51].

Moreover, CB2 receptors were reported to play a role in reducing oxidative stress markers in hydrogen peroxide stimulated cultured RAW 264.7.7 macrophages [52].

p53 is an oncoprotein, potentially stimulate cell growth arrest and apoptosis and is upregulated in many liver diseases, ranging from fatty liver disease to HCC [53]. p53-dependent mitochondrial stress is found to induce apoptosis associated with increased Bax and Bcl2 in bile acid-induced liver toxicity in both in-vivo and in-vitro [54], [55]. It is also associated with increased lipid peroxidation and mitochondrial stress in CCL4 rats [56], [57], [58]. Likely, BDL in the present study showed a marked increase in the apoptotic process with strong cytoplasmic immunoreactions for p53. However, daily treatment with AM1241 produced a significant reduction in expression of p53 as compared to both BDL and silymarin groups. Previous studies also reported that 2-AG-mediated CB2 signalling by MAGL inhibition protects against hepatocyte apoptosis elicited by acute liver injury [59]. However, CB2 receptors are not expressed inside the hepatocytes, their impact on modulation of the proinflammatory cytokines and oxidative stress may explain an indirect and paracrine effect on hepatocyte apoptosis.

A heated debate raged for years as regard to the role of oval/progenitor cells in liver regeneration which is capable of generating both hepatocytes and biliary cells following BDL [60], [61], [62]. Our results showed that sham rats showed negative expression of CD34 and α-FP by immunostaining and gene expression with no detection of oval cells by H&E. However, BDL and silymarin groups showed moderate expression of these oval cell markers and its gene expression with observed oval cells by H&E. Interestingly, treatment with AM1241 produced a significant expression of α-FP and CD34 immunoreaction and enhanced its gene expression with more detected oval cells by H&E as compared to both BDL and silymarin group. Moreover, certain bile acids and growth factors were found to be a potent stimulator of progenitor profile of mesenchymal cells [63] where others reported that kupffer cells modulation initiate oval cells stimulation [64]. Whether CB2-dependent modulation of kupffer cells activity and growth factors plays a role in progenitor cells stimulation would be further investigated. Moreover, CB2 receptors were previously reported to promote neural and cardiac progenitor cells regeneration. Palazuelos, Aguado [65] reported that stimulation of CB2 receptors enhanced the proliferation of neural progenitor cells, both in vitro and in vivo while Wang, Ma [66] reported that AM1241 promoted proliferation of ki-67 immunostained cardiac progenitor cells (CPCs) and encouraged cardiomyocyte regeneration in post-myocardial infarction.

In conclusion, our data demonstrate that CB2 receptors reduce liver injury and fibrogenesis following bile duct ligation with explaining new distinct mechanisms originating from hepatic myofibroblasts and immune cells. These include IL-10 dependent inhibition of inflammatory mediators, reduction of lipid peroxidation inside hepatic cells and inhibition of hepatocyte p53-dependent apoptosis. Besides, CB2 receptors are shown to possess a regenerative effect through stimulation of HPCs stimulation. These results suggest that CB2 agonists display potent hepatoregenrative properties, in addition to their antifibrogenic effects.

References

1. Caballería L, Pera G, Arteaga I, Rodríguez L, Alumà A, Morillas RM, de la Ossa N, Díaz A, Expósito C, Miranda D, Sánchez C. High prevalence of liver fibrosis among European adults with unknown liver disease: a population-based study. Clinical Gastroenterology and Hepatology. 2018; 16(7):1138-45. https://doi.org/10.1016/j.cgh.2017.12.048 PMid:29452268

2. Zois CD, Baltayiannis GH, Karayiannis P, Tsianos EV. Systematic review: hepatic fibrosis–regression with therapy. Alimentary pharmacology & therapeutics. 2008; 28(10):1175-87.

https://doi.org/10.1111/j.1365-2036.2008.03840.x PMid:18761707

3. Purohit V, Rapaka R, Shurtleff D. Role of cannabinoids in the development of fatty liver (steatosis). AAPS J. 2010; 12(2):233-7. https://doi.org/10.1208/s12248-010-9178-0 PMid:20204561 PMCid:PMC2844512

4. Mallat A, Lotersztajn S. Cannabinoid receptors as novel therapeutic targets for the management of non-alcoholic steatohepatitis. Diabetes & metabolism. 2008; 34(6):680-4. https://doi.org/10.1016/S1262-3636(08)74604-4

5. Jeong WI, Osei-Hyiaman D, Park O, Liu J, Bátkai S, Mukhopadhyay P, Horiguchi N, Harvey-White J, Marsicano G, Lutz B, Gao B. Paracrine activation of hepatic CB1 receptors by stellate cell-derived endocannabinoids mediates alcoholic fatty liver. Cell metab. 2008; 7(3):227-35. https://doi.org/10.1016/j.cmet.2007.12.007 PMid:18316028

6. Julien B, Grenard P, Teixeira-Clerc F, Van Nhieu JT, Li L, Karsak M, Zimmer A, Mallat A, Lotersztajn S. Antifibrogenic role of the cannabinoid receptor CB2 in the liver. Gastroenterology. 2005; 128(3):742-55. https://doi.org/10.1053/j.gastro.2004.12.050 PMid:15765409

7. Lotersztajn S, Teixeira-Clerc F, Julien B, Deveaux V, Ichigotani Y, Manin S, Tran-Van-Nhieu J, Karsak M, Zimmer A, Mallat A. CB2 receptors as new therapeutic targets for liver diseases. British journal of pharmacology. 2008; 153(2):286-9. https://doi.org/10.1038/sj.bjp.0707511 PMid:17952109 PMCid:PMC2219531

8. Teixeira-Clerc F, Belot MP, Manin S, Deveaux V, Cadoudal T, Chobert MN, Louvet A, Zimmer A, Tordjmann T, Mallat A, Lotersztajn S. Beneficial paracrine effects of cannabinoid receptor 2 on liver injury and regeneration. Hepatology. 2010; 52(3):1046-59. https://doi.org/10.1002/hep.23779 PMid:20597071 PMCid:PMC3246453

9. Bátkai S, Osei-Hyiaman D, Pan H, El-Assal O, Rajesh M, Mukhopadhyay P, Hong F, Harvey-White J, Jafri A, Hasko G, Huffman JW. Cannabinoid-2 receptor mediates protection against hepatic ischemia/reperfusion injury. The FASEB Journal. 2007; 21(8):1788-800. https://doi.org/10.1096/fj.06-7451com PMid:17327359 PMCid:PMC2228252

10. Rajesh M, Mukhopadhyay P, Bátkai S, Haskó G, Liaudet L, Huffman JW, Csiszar A, Ungvari Z, Mackie K, Chatterjee S, Pacher P. CB2-receptor stimulation attenuates TNF-α-induced human endothelial cell activation, transendothelial migration of monocytes, and monocyte-endothelial adhesion. American journal of physiology. Heart and circulatory physiology. 2007; 293(4):H2210-8. https://doi.org/10.1152/ajpheart.00688.2007 PMid:17660390 PMCid:PMC2229632

11. Mukhopadhyay P, et al. Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro. Am J Physiol Heart Circ Physiol. 2009. 296(5):H1466-83. https://doi.org/10.1152/ajpheart.00795.2008 PMid:19286953 PMCid:PMC2685360

12. Moon KH, Hood BL, Mukhopadhyay P, Rajesh M, Abdelmegeed MA, Kwon YI, Conrads TP, Veenstra TD, Song BJ, Pacher P. Oxidative inactivation of key mitochondrial proteins leads to dysfunction and injury in hepatic ischemia reperfusion. Gastroenterology. 2008; 135(4):1344-57. https://doi.org/10.1053/j.gastro.2008.06.048 PMid:18778711 PMCid:PMC2597302

13. Abe Y, Hines IN, Zibari G, Pavlick K, Gray L, Kitagawa Y, Grisham MB. Mouse model of liver ischemia and reperfusion injury: method for studying reactive oxygen and nitrogen metabolites in vivo. Free Radical Biology and Medicine. 2009; 46(1):1-7. https://doi.org/10.1016/j.freeradbiomed.2008.09.029 PMid:18955130 PMCid:PMC2740994

14. Hellerbrand C, Schattenberg JM, Peterburs P, Lechner A, Brignoli R. The potential of silymarin for the treatment of hepatic disorders. Clinical Phytoscience. 2017; 2(1):7. https://doi.org/10.1186/s40816-016-0019-2

15. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. Animal research: reporting in vivo experiments: the ARRIVE guidelines. British journal of pharmacology. 2010; 160(7):1577-9. https://doi.org/10.1111/j.1476-5381.2010.00872.x PMid:20649561 PMCid:PMC2936830

16. Kountouras J, Billing BH, Scheuer PJ. Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. British journal of experimental pathology. 1984; 65(3):305. PMid:6743531 PMCid:PMC2040968

17. Struck MB, Andrutis KA, Ramirez HE, Battles AH. Effect of a short-term fast on ketamine–xylazine anesthesia in rats. Journal of the American Association for Laboratory Animal Science. 2011; 50(3):344-8. PMid:21640029 PMCid:PMC3103284

18. Aller MA, Arias JL, Prieto I, Arias J. A suitable microsurgical method for obstructive cholestasis in the rat. Protocol Exchange. 2010. https://doi.org/10.1038/nprot.2010.56

19. Fahmy Ahmed A, Fouad Mahmoud M, Arafat Ouf M, El-Fathaah EA. Aminoguanidine potentiates the hepatoprotective effect of silymarin in CCL4 treated rats. Annals of hepatology. 2016; 10(2):207-15.

20. Barutta F, Piscitelli F, Pinach S, Bruno G, Gambino R, Rastaldi MP, Salvidio G, Di Marzo V, Perin PC, Gruden G. Protective role of cannabinoid receptor type 2 in a mouse model of diabetic nephropathy. Diabetes. 2011; 60(9):2386-96. https://doi.org/10.2337/db10-1809 PMid:21810593 PMCid:PMC3161308

21. Babson AL, Greeley SJ, Coleman CM, Phillips GE. Phenolphthalein monophosphate as a substrate for serum alkaline phosphatase. Clinical chemistry. 1966; 12(8):482-90. PMid:5917856

22. Doumas BT, Perry BW, Sasse EA, Straumfjord JV. Standardization in bilirubin assays: evaluation of selected methods and stability of bilirubin solutions. Clinical chemistry. 1973; 19(9):984-93. PMid:4744825

23. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. American journal of clinical pathology. 1957; 28(1):56-63. https://doi.org/10.1093/ajcp/28.1.56 PMid:13458125

24. Yuksel Y, Guven M, Kaymaz B, Sehitoglu MH, Aras AB, Akman T, Tosun M, Cosar M. Effects of aloe vera on spinal cord Ischemia–Reperfusion injury of rats. Journal of Investigative Surgery. 2016; 29(6):389-98. https://doi.org/10.1080/08941939.2016.1178358 PMid:27142763

25. Atli O, Baysal M, Aydogan-Kilic G, Kilic V, Ucarcan S, Karaduman B, Ilgin S. Sertraline-induced reproductive toxicity in male rats: evaluation of possible underlying mechanisms. Asian journal of andrology. 2017; 19(6):672. https://doi.org/10.4103/1008-682X.192637 PMid:27976631 PMCid:PMC5676427

26. Li J, Yan HT, Che JX, Bai SR, Qiu QM, Ren L, Pan F, Sun XQ, Tian FZ, Li DX, Tang LJ. Effects of neurolytic celiac plexus block on liver regeneration in rats with partial hepatectomy. PloS one. 2013; 8(9):e73101. https://doi.org/10.1371/journal.pone.0073101 PMid:24039865 PMCid:PMC3764180

27. Lee K, Lee B, Lee MH, Kim B, Chinannai KS, Ham I, Choi HY. Effect of Ampelopsis Radix on wound healing in scalded rats. BMC complementary and alternative medicine. 2015; 15(1):213. https://doi.org/10.1186/s12906-015-0751-z PMid:26152211 PMCid:PMC4495638

28. Naidu S, Peterson ML, Spear BT. Alpha-fetoprotein related gene (ARG): a new member of the albumin gene family that is no longer functional in primates. Gene. 2010; 449(1-2):95-102. https://doi.org/10.1016/j.gene.2009.08.014 PMid:19733224 PMCid:PMC2799041

29. Omori N, Omori M, Evarts RP, Teramoto T, Miller MJ, Hoang TN, Thorgeirsson SS. Partial cloning of rat CD34 cDNA and expression during stem cell-dependent liver regeneration in the adult rat. Hepatology. 1997; 26(3):720-7. https://doi.org/10.1002/hep.510260325 PMid:9303503

30. Bancroft JD, Stevens A. Theory and Practice of Histological Techniques. Churchill Livingstone, 1996.

31. Coons AH, Creech HJ, Jones RN. Immunological properties of

an antibody containing a fluorescent group. Proceedings of the Society for Experimental Biology and Medicine. 1941; 47(2):200-2. https://doi.org/10.3181/00379727-47-13084P

32. Filimonova G, Tokin II, Tokin IB, Hussar P. An assessment of morphometric analysis in liver biopsy specimens of patients with chronic hepatitis C. Papers on Anthropology. 2010; (19):69-80.

33. Curtis MJ, Abernethy DR. Advice on statistical analysis, and new journal guidance for experimental design and analysis. Pharmacology research & perspectives. 2015; 3(1):e00095. https://doi.org/10.1002/prp2.95 PMid:25692014 PMCid:PMC4317227

34. Deveaux V, Cadoudal T, Ichigotani Y, Teixeira-Clerc F, Louvet A, Manin S, Tran-Van Nhieu J, Belot MP, Zimmer A, Even P, Cani PD. Cannabinoid CB2 receptor potentiates obesity-associated inflammation, insulin resistance and hepatic steatosis. PLoS One. 2009; 4(6):e5844. https://doi.org/10.1371/journal.pone.0005844 PMid:19513120 PMCid:PMC2688760

35. Tarcin O, Basaranoglu M, Tahan V, Tahan G, Sücüllü I, Yilmaz N, Sood G, Snyder N, Hilman G, Celikel C, Tözün N. Time course of collagen peak in bile duct-ligated rats. BMC gastroenterology. 2011; 11(1):45. https://doi.org/10.1186/1471-230X-11-45 PMid:21527001 PMCid:PMC3117813

36. Copple BL, Jaeschke H, Klaassen CD. Oxidative stress and the pathogenesis of cholestasis. Semin Liver Dis. 2010; 30(2):195-204. https://doi.org/10.1055/s-0030-1253228 PMid:20422501

37. Gujral JS, Farhood A, Bajt ML, Jaeschke H. Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct–ligated mice. Hepatology. 2003; 38(2):355-63. https://doi.org/10.1053/jhep.2003.50341 PMid:12883479

38. Friedman SL. Liver fibrosis–from bench to bedside. Journal of hepatology. 2003; 38:38-53. https://doi.org/10.1016/S0168-8278(02)00429-4

39. Mu-oz-Luque J, Ros J, Fernández-Varo G, Tugues S, Morales-Ruiz M, Alvarez CE, Friedman SL, Arroyo V, Jiménez W. Regression of fibrosis after chronic stimulation of cannabinoid CB2 receptor in cirrhotic rats. Journal of Pharmacology and Experimental Therapeutics. 2008; 324(2):475-83. https://doi.org/10.1124/jpet.107.131896 PMid:18029545 PMCid:PMC2887659

40. Louvet A, Teixeira-Clerc F, Chobert MN, Deveaux V, Pavoine C, Zimmer A, Pecker F, Mallat A, Lotersztajn S. Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating Kupffer cell polarization in mice. Hepatology. 2011; 54(4):1217-26. https://doi.org/10.1002/hep.24524 PMid:21735467

41. Guillot A, Hamdaoui N, Bizy A, Zoltani K, Souktani R, Zafrani ES, Mallat A, Lotersztajn S, Lafdil F. Cannabinoid receptor 2 counteracts interleukin-17-induced immune and fibrogenic responses in mouse liver. Hepatology. 2014; 59(1):296-306. https://doi.org/10.1002/hep.26598 PMid:23813495

42. Jackson AR, Nagarkatti P, Nagarkatti M. Anandamide attenuates Th-17 cell-mediated delayed-type hypersensitivity response by triggering IL-10 production and consequent microRNA induction. PloS one. 2014; 9(4):e93954. https://doi.org/10.1371/journal.pone.0093954 PMid:24699635 PMCid:PMC3974854

43. Sun YY, Li XF, Meng XM, Huang C, Zhang L, Li J. Macrophage phenotype in liver injury and repair. Scandinavian journal of immunology. 2017; 85(3):166-74. https://doi.org/10.1111/sji.12468 PMid:27491503

44. Naito Y, Takagi T, Higashimura Y. Heme oxygenase-1 and anti-inflammatory M2 macrophages. Archives of biochemistry and biophysics. 2014; 564:83-8. https://doi.org/10.1016/j.abb.2014.09.005 PMid:25241054

45. Hull TD, Agarwal A, George JF. The mononuclear phagocyte system in homeostasis and disease: a role for heme oxygenase-1. Antioxidants & redox signaling. 2014; 20(11):1770-88. https://doi.org/10.1089/ars.2013.5673 PMid:24147608 PMCid:PMC3961794

46. Guo B. IL-10 modulates Th17 pathogenicity during autoimmune diseases. Journal of clinical & cellular immunology. 2016; 7(2):400. https://doi.org/10.4172/2155-9899.1000400 PMid:27308096 PMCid:PMC4905582

47. Huber S, Gagliani N, Esplugues E, O'Connor Jr W, Huber FJ, Chaudhry A, Kamanaka M, Kobayashi Y, Booth CJ, Rudensky AY, Roncarolo MG. Th17 cells express interleukin-10 receptor and are controlled by Foxp3− and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. Immunity. 2011; 34(4):554-65. https://doi.org/10.1016/j.immuni.2011.01.020 PMid:21511184 PMCid:PMC3113617

48. Gu Y, Yang J, Ouyang X, Liu W, Li H, Yang J, Bromberg J, Chen SH, Mayer L, Unkeless JC, Xiong H. Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. European journal of immunology. 2008; 38(7):1807-13. https://doi.org/10.1002/eji.200838331 PMid:18506885 PMCid:PMC2733944

49. Pacher P, Hasko G. Endocannabinoids and cannabinoid receptors in ischaemia–reperfusion injury and preconditioning. British journal of pharmacology. 2008; 153(2):252-62. https://doi.org/10.1038/sj.bjp.0707582 PMid:18026124 PMCid:PMC2219536

50. Jaeschke H. Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2006; 290(6):G1083-8. https://doi.org/10.1152/ajpgi.00568.2005 PMid:16687579

51. Wang Y, Mukhopadhyay P, Cao Z, Wang H, Feng D, Haskó G, Mechoulam R, Gao B, Pacher P. Cannabidiol attenuates alcohol-induced liver steatosis, metabolic dysregulation, inflammation and neutrophil-mediated injury. Scientific reports. 2017; 7(1):12064. https://doi.org/10.1038/s41598-017-10924-8 PMid:28935932 PMCid:PMC5608708

52. Giacoppo S, Gugliandolo A, Trubiani O, Pollastro F, Grassi G, Bramanti P, Mazzon E. Cannabinoid CB2 receptors are involved in the protection of RAW264. 7 macrophages against the oxidative stress: an in vitro study. European journal of histochemistry: EJH. 2017; 61(1):2749. https://doi.org/10.4081/ejh.2017.2749 PMid:28348416 PMCid:PMC5289301

53. Krstic J, Galhuber M, Schulz T, Schupp M, Prokesch A. P53 as a dichotomous regulator of liver disease: The dose makes the medicine. International journal of molecular sciences. 2018; 19(3):921. https://doi.org/10.3390/ijms19030921 PMid:29558460 PMCid:PMC5877782

54. Castro RE, Amaral JD, Solá S, Kren BT, Steer C, Rodrigues CM. Differential regulation of cyclin D1 and cell death by bile acids in primary rat hepatocytes. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2007; 293(1):G327-34. https://doi.org/10.1152/ajpgi.00093.2007 PMid:17431217

55. Ferreira DM, Afonso MB, Rodrigues PM, Simão AL, Pereira DM, Borralho PM, Rodrigues CM, Castro RE. c-Jun N-terminal kinase 1/c-Jun activation of the p53/microRNA 34a/sirtuin 1 pathway contributes to apoptosis induced by deoxycholic acid in rat liver. Molecular and cellular biology. 2014; 34(6):1100-20. https://doi.org/10.1128/MCB.00420-13 PMid:24421392 PMCid:PMC3958031

56. Tian XF, Ji FJ, Zang HL, Cao H. Activation of the miR-34a/SIRT1/p53 signaling pathway contributes to the progress of liver fibrosis via inducing apoptosis in hepatocytes but not in HSCs. PLoS One. 2016; 11(7):e0158657. https://doi.org/10.1371/journal.pone.0158657 PMid:27387128 PMCid:PMC4936740

57. Eltablawy NA, Ogaly HA. Responsiveness of p53 expression and genetic mutation to CCl4-induced DNA damage in rat's liver. International Journal of Genomics and Proteomics, ISSN. 2014:0976-4887.

58. Guo XL, Liang B, Wang XW, Fan FG, Jin J, Lan R, Yang JH, Wang XC, Jin L, Cao Q. Glycyrrhizic acid attenuates CCl4-induced hepatocyte apoptosis in rats via a p53-mediated pathway. World Journal of Gastroenterology: WJG. 2013; 19(24):3781-91.

https://doi.org/10.3748/wjg.v19.i24.3781 PMid:23840116 PMCid:PMC3699029

59. Cao Z, Mulvihill MM, Mukhopadhyay P, Xu H, Erdélyi K, Hao E, Holovac E, Haskó G, Cravatt BF, Nomura DK, Pacher P. Monoacylglycerol lipase controls endocannabinoid and eicosanoid signaling and hepatic injury in mice. Gastroenterology. 2013; 144(4):808-17. https://doi.org/10.1053/j.gastro.2012.12.028 PMid:23295443 PMCid:PMC3608818

60. Lotowska JM, Sobaniec-Lotowska ME, Lebensztejn DM, Daniluk U, Sobaniec P, Sendrowski K, Daniluk J, Reszec J, Debek W. Ultrastructural Characteristics of Rat Hepatic Oval Cells and Their Intercellular Contacts in the Model of Biliary Fibrosis: New Insights into Experimental Liver Fibrogenesis. Gastroenterology research and practice. 2017; 2017:2721547. https://doi.org/10.1155/2017/2721547 PMid:28769978 PMCid:PMC5523291

61. Roskams TA, Libbrecht L, Desmet VJ. Progenitor cells in diseased human liver. Semin Liver Dis. 2003; 23(4): 385-96. https://doi.org/10.1055/s-2004-815564 PMid:14722815

62. Lowes KN, Croager EJ, Olynyk JK, Abraham LJ, Yeoh GC. Oval cell-mediated liver regeneration: Role of cytokines and growth factors. Journal of gastroenterology and hepatology. 2003; 18(1):4-12. https://doi.org/10.1046/j.1440-1746.2003.02906.x PMid:12519217

63. Sawitza I, Kordes C, Götze S, Herebian D, Häussinger D. Bile acids induce hepatic differentiation of mesenchymal stem cells. Scientific reports. 2015; 5:13320. https://doi.org/10.1038/srep13320 PMid:26304833 PMCid:PMC4548444

64. Olynyk JK, Yeoh GC, Ramm GA, Clarke SL, Hall PD, Britton RS, Bacon BR, Tracy TF. Gadolinium chloride suppresses hepatic oval cell proliferation in rats with biliary obstruction. The American journal of pathology. 1998; 152(2):347-52. PMid:9466559 PMCid:PMC1857969

65. Palazuelos J, Aguado T, Egia A, Mechoulam R, Guzmán M, Galve-Roperh I, Palazuelos J, Aguado T, Egia A, Mechoulam R, Guzmán M. Non-psychoactive CB2 cannabinoid agonists stimulate neural progenitor proliferation. The FASEB Journal. 2006; 20(13):2405-7. https://doi.org/10.1096/fj.06-6164fje PMid:17015409

66. Wang Y, Ma S, Wang Q, Hu W, Wang D, Li X, Su T, Qin X, Zhang X, Ma K, Chen J. Effects of cannabinoid receptor type 2 on endogenous myocardial regeneration by activating cardiac progenitor cells in mouse infarcted heart. Science China Life Sciences. 2014; 57(2):201-8. https://doi.org/10.1007/s11427-013-4604-z PMid:24430557