

Cannabinoid receptor type 2 agonist JWH-133 deteriorates the liver toxicity induced by cypermethrin*

Hidayet TUTUN^{1**}, Emine BAYDAN²

¹Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey

²Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

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Abstract: Cannabinoid receptor 2 (CB2) has a role in the pathology of some liver diseases. Cypermethrin (CYP) is a synthetic pyrethroid insecticide used commonly for the control of pests and vectors, and for protection of foodstuffs. In this study, the involvement of CB2 in CYP-induced liver damage in rats was investigated. CYP was applied to rats orally at a dose of 125 mg/kg bw per day for 14 days. CB2 agonist JWH-133 was administered by intraperitoneal injection at a dose of 3 mg/kg bw per day for the last 4 days of CYP toxicity. JWH-133 deteriorated the liver damage induced by CYP. JWH-133 increased serum hepatic enzyme activities (aspartate aminotransferase, alanine aminotransferase) in rats given CYP. However, it decreased lipid peroxidation. There were no differences in the levels of hepatic CB2 mRNA among the groups. CB2 receptors were expressed in a small amount in normal liver tissue. In contrast, the expression levels of CB2 in CYP-treated rats were increased in fibrocyte/fibroblast, bile duct epithelial cells, Kupffer cells, and mast cells. In conclusion, CB2 was upregulated in the liver exposed to CYP, predominantly in hepatic fibrogenic cells. JWH-133 enhanced the CYP-induced liver damage by receptor mediated and/or nonreceptor-mediated mechanisms. CB2 antagonists may reduce the damage. The results of the study show that CB2 might be involved in the physiopathological process of CYP-induced liver injury and justify the need for further study.

Key words: Cannabinoid receptor type 2, CB2, cypermethrin, JWH-133, liver, rat

1. Introduction

CYP is a synthetic type II pyrethroid insecticide widely used for the control of ectoparasites of domestic animals and home pest control owing to its high insecticidal potential and lower mammalian toxicity (1). CYP shows its effect via the voltage-dependent Na⁺ and Ca²⁺ channels in the membrane of nerve cells (2–4). There is an increasing amount of evidence suggesting toxic effects on nontarget organisms in chronic exposure of CYP (5,6). Several studies have shown that CYP causes oxidative stress, biochemical changes such as increase in activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH); elevation in levels of thiobarbituric acid reactive substances (TBARS), a decrease in activities of the antioxidant enzymes (7,8), and histopathological changes such as vacuolar degeneration, enlarged sinusoid,

vacuole formation in hepatocytes, pleomorphism in the nucleus, congestion, necrosis, hepatocellular hypertrophy, multinucleated hepatocytes, mononuclear cell infiltration (MCI), and increase in Kupffer cell numbers (2,6).

Marijuana or cannabis, whose main active ingredient is delta-9-tetrahydrocannabinol, activates two G protein-coupled membrane receptors, named CB1 and CB2 (9–11). CB1 is mainly expressed in the nervous system and is less expressed in various peripheral tissues such as the heart, gut, liver, and in cells such as endothelial cells and adipocytes. In comparison, CB2 is largely expressed in immune cells, tonsils, spleen, and testis. Moreover, it has been observed to be expressed to a lesser extent in other tissues and in cells like hepatic myofibroblasts (12).

Recent studies have shown the dysregulation of endocannabinoid system in experimental models of bowel, liver, and heart diseases. Evidence suggests that

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** Correspondence: hidayetutun@gmail.com

selective CB2 agonists may protect against hepatic, cerebral, and myocardial ischemia/reperfusion (I/R) injuries (13,14). Although CB1 and CB2 are expressed at a low level in normal livers, they are markedly upregulated in experimental liver injury and liver cirrhosis of various etiologies. Upregulation occurs in spindle-shaped cells, inflammatory cells, and ductular proliferating cells. CB2 is also expressed in hepatocytes of nonalcoholic steatosis and steatohepatitis, and in activated hepatic stellate cells and hepatic myofibroblasts within fibrous septa (15,16). Numerous studies have been done on the effect of endocannabinoid system on liver diseases. Consequently, these studies have suggested that CB1 antagonism or CB2 activation may provide a significant therapeutic benefit (12,14). The purpose of this study was to investigate the involvement of CB2 in CYP-induced liver damage and the effects of JWH-133 on liver injury in rats.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (180–240 g) were used (Ankara University Farms, Turkey). The rats were housed under a 12-h light/dark cycle at a constant temperature and humidity and given access to food and water ad libitum. The study was performed according to the European Community Council Directive (2010/63/EU) and approved by the Ankara University Animal Experiments Local Ethic Board (2014-16-104).

2.2. Experimental design

CYP (Alfa Aesar) was dissolved in 1 mL of corn oil at a dose of 125 mg/kg body weight. JWH-133 (Axon) was freshly prepared in a vehicle solution containing 1 drop of Tween 20 (Sigma) in 0.1 mL of dimethyl sulfoxide (Sigma) and then diluted in NaCl 0.9%. After acclimatization, six rats were sacrificed on the first day as negative controls (negative control group, NCG). Then the rats were divided into four sets. Group 1 served as controls (only vehicle and corn oil). CYP was administered orally through gavage to groups 2 and 3 for 14 days. The rats in groups 3 and 4 received JWH-133 (3 mg/kg, ip) for the last 4 days. Body weights were measured daily. The rats were sacrificed after 14 days. Blood samples were taken by cardiac puncture for liver enzymes. Liver tissues were taken, weighed, and either fixed in buffered formalin, or rapidly frozen in dry ice. All samples were stored at -80°C until they were used.

2.3. Liver function tests

The blood samples were centrifuged to get serum at 4000 rpm for 10 min and the serum samples were maintained at -80°C until they were analyzed. The activity of AST, ALT, and alkaline phosphatase (ALP), and the level of glucose were measured in the serum samples collected using a chemistry analyzer system (Cobas-C501, Roche Diagnostics).

2.4. Oxidative stress parameters

Liver tissues were dissected and separately homogenized in ice-cold homogenization buffer (86.0 g of saccharose, 0.372 g of EDTA, 0.606 g of Tris in 1 L tridistilled water, pH 7.4) for glutathione peroxidase (GPx), homogenization buffer (50 mmol/L and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 1 L tridistilled water, 0.5% Tween 20) for superoxide dismutase (SOD), and RIPA buffer (Cayman; cat: 100010263) for TBARS by using an ultrasound homogenizer (Bandelin, Sonoplus UW 3100). The homogenates were centrifuged at $12,000 \times g$ for 10 min at 4°C . GPx was measured by the method of Paglia and Valentine (17) using a Glutathione Peroxidase Kit (Cayman, 703102), SOD was measured by the method of Sun et al. (18) using a Superoxide Dismutase Assay Kit (Cayman, 706002), TBARS was measured by the method of Ohkawa et al. (19) using TBARS Assay Kit (Cayman, 100009055) in supernatants collected, and assays were made according to their own protocol. Moreover, the protein content of each extract was determined using a Total Protein Kit (Erba; cat. BLT00054) in the autoanalyzer (Erba XL 600, Meinheim, Germany).

2.5. Liver histopathology

Part of the liver tissue was fixed in 10% neutral-buffered formalin for 24 h, routinely processed, and embedded in paraffin. The paraffin blocks were cut in 5- μm -thick sections. They were used for immunostaining and histopathological examination. Sections were stained with hematoxylin/eosin (HE) for routine examination. Liver sections were assessed by a veterinary pathologist blinded to study conditions. Slides were examined using a light microscope and photographs were taken at the Veterinary Department of Pathology, Ankara University, using an automated digital camera system (Olympus CH30/Ch40, Japan). Parenchyma degeneration and necrosis were scored according to severity as negative (-), weak (+), moderate (++) , and severe (+++) (2).

2.6. Immunohistochemistry (IHC)

After liver sections were deparaffinized at 55°C for 30 min during the time they were in the oven, they were rehydrated in Tris buffered saline (TBS) (Abcam, ab64248). Sections underwent antigen retrieval to unmask antigens for 20 min (10% citrate buffer). Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide (Abcam, ab64261) pretreatment for 20 min in the dark, and then sections were preincubated in serum blocking buffer (Abcam, ab64261) for 10 min, stained with the primary antibody (Abcam, rabbit polyclonal anti-rat CB2, ab45942), diluted 1:100 in antibody diluent (Abcam, ab64211), and incubated overnight at 4°C . After rinsing 3 times for 5 min in TBS, sections were incubated with biotinylated secondary antibody (Abcam, rabbit specific HRP/AEC, ab64261) for 30 min at room temperature. Sections were rinsed 3 times for 5 min and antigen

visualization was achieved with streptavidin (Streptavidin HRP/AEC, ab64261) for 30 min at room temperature. After they were rinsed 3 times for 4 min, sections were incubated with AEC (3-amino-9 ethylcarbazole) (Thermo Scientific, ta125ha) for about 1 min and counterstained with hematoxylin (Abcam, ab128990) for about 40 s. CB2 positive staining was visualized and photographed using a digital microscope (Leica DM 2500). As negative control, immunostaining was carried out without the first antibody. As positive control, immunostaining was performed on a spleen. Reactions were scored in terms of severity of immunostained cells and areas as negative (-), very weak (+), weak (++) , moderate (+++) , and strong (++++) (20). Two independent observers assessed the immunohistochemical reaction.

2.7. RNA extraction, transcription, and amplification

A commercial kit (Thermo Scientific GeneJET RNA Purification Kit, Cat. K0731) was used for total RNA extraction from the liver. Quantitative real-time PCR (QRT-PCR) was performed on a QIAGEN Rotor-Gene P. cDNA synthesis was carried out from 1 µg of total RNA with random hexamers using RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific). QRT-PCR primers (sense, 5'-TGACTGCTGTTGACCGATAACCT-3'; antisense, 5'-GCCGAGAGGACCCACATG-3'), and probe (5'-Fam-CTACCTACAAAGCTCTCGTCACCCGTGG-Tamra-3') were designed by Beltramo et al. (21) according to rat CB2 gene (GenBank: AF286721). The positive control was manufactured synthetically by a commercial firm (SENTEGEN). QRT-PCR was carried out with Maxima Probe qPCR Master Mix (Thermo Scientific). All samples were run in duplicate. The reaction was carried out with the following steps: 2 min at 50 °C; 10 min at 95 °C, once, and 15 s at 95 °C and 1 min at 60 °C for 40 cycles (21). Fluorescent emission for each sample was measured after each annealing step. The positive control was employed in each cycle as a standard for quantification.

2.8. Statistical analyses

All values are means ± SEM. All variables obtained were examined using the Shapiro-Wilk parametric test hypotheses for normality, and the Levene test for homogeneity of variance. Statistical analysis was performed using one-way analysis of variance (ANOVA). The Duncan test was used as a post-hoc test for significant differences between the groups. SPSS 14.01 was used.

3. Results

3.1. Morphometric parameters

There were statistically significant differences in changes of body weight between the groups ($P < 0.001$). The lowest weight gain occurred in group 3. JWH-133 significantly reduced body weight gain in groups 3 and 4. There were

no significant differences in relative liver weight between the groups (Table 1).

3.2. Clinical signs

Typical signs of CYP toxicity, including hypersalivation, hypersensitivity, hunched back, scratching, ataxia, hind limb paralysis, and convulsions were observed in rats treated with CYP, which began 2 h after dosing. No clinical signs were observed in groups 1 and 4. Activation of CB2 did not alleviate the typical signs of CYP toxicity in group 3.

3.3. Hepatic function tests

Compared to control group, the activities of serum AST ($P > 0.05$) and ALT ($P < 0.05$) increased while the activities of serum ALP ($P > 0.05$) decreased in group 2. There was a reduction in the activity of serum ALP and an increase in the activities of serums ALT and AST in group 4 when compared to the control. Serum AST and ALT levels were higher in group 3 in comparison with the control (Table 2). Overall, these data indicate that JWH-133 aggravates liver damage induced by CYP.

3.4. Lipid peroxidation

Malondialdehyde (MDA) formation was measured as TBARS material. An increase in MDA level ($P > 0.05$) and a reduction in SOD ($P > 0.05$) and GPx ($P < 0.05$) activity were observed in group 2 as compared to the control. There was a significant decrease in SOD ($P < 0.05$) activity, a small increase in GPx ($P > 0.05$) activity, and a reduction in the level of MDA in group 3, as compared to group 2. Level of MDA, SOD, and GPx activities underwent a reduction in group 4. SOD/GPx activity rate was higher in group 2 ($P < 0.05$) in comparison with the other groups (Table 3).

3.5. Immunohistochemistry

The expression of CB2 was examined by IHC with a polyclonal antibody against rat CB2 on tissue sections obtained from rat livers in the groups. Very weak immunoreactivity for CB2 was detected in the liver in the control group. In contrast, livers obtained from CYP-treated groups showed a strong CB2 immunostaining in vena centralis and trias hepatis. CB2 expression was found in fibroblast, biliary duct epithelium, Kupffer, and mast cells (Figure 1). A scoring of the distribution of CB2 expressed in livers as type of cells is given in Table 4.

CB2 protein was not detected in hepatocytes. Negative and positive control staining were performed in rat spleen (Figure 1). CB2 protein was expressed during subacute CYP toxicity in the rat liver.

3.6. Hepatic histopathology

Histopathologic examination of the rats in group 1 and NGC showed normal hepatic histology (Figure 2a). Histopathological alterations were observed in rat livers in groups 2 and 3. Alterations ranging from moderate degeneration (++) to necrotic were noted in group 2

Table 1. Effects of CYP and JWH-133 on the relative body and liver weights of rats in the groups.

Parameters	Group 1 (n: 7)	Group 2 (n: 9)	Group 3 (n: 9)	Group 4 (n: 7)
Initial weight (g)	192.14 ± 31.38	195.77 ± 21.46	179.33 ± 19.95	175.71 ± 13.53
Last weight (g)	233.71 ± 28.48	217.00 ± 19.30	193.11 ± 19.74	205.71 ± 19.77
Changes in weight (g)	41.57 ± 2.82 ^a	21.22 ± 2.17 ^c	13.77 ± 1.49 ^d	30.00 ± 2.71 ^b
Liver weight (g)	10.61 ± 1.20	11.33 ± 1.32	9.85 ± 1.87	9.91 ± 1.09
Relative liver weight	0.0454 ± 0.002	0.0521 ± 0.003	0.051 ± 0.011	0.048 ± 0.003

Values are given as mean ± SEM. (a,b,c,d). Different characters indicate statistically significant differences in the same line (P < 0.001). Group 1: control; group 2: CYP-treated rats; group 3: CYP + JWH-133-treated rats; group 4: JWH-133-treated rats.

Table 2. Activities of liver enzymes and levels of glucose in the blood serum of rats.

	NGC (n: 6)	Group 1 (n: 6)	Group 2 (n: 7)	Group 3 (n: 7)	Group 4 (n: 6)
ALP*	266.57 ± 5.32 ^{ab}	280.40 ± 31.50 ^a	269.14 ± 18.94 ^{ab}	260.71 ± 10.87 ^{ab}	222.33 ± 19.15 ^b
AST**	129.57 ± 12.88 ^b	131 ± 5.48 ^b	149.42 ± 8.41 ^{ab}	175.71 ± 18.07 ^a	148.16 ± 7.91 ^{ab}
ALT***	63.86 ± 0.96 ^b	72.16 ± 4.61 ^b	88.42 ± 3.60 ^a	88.85 ± 1.97 ^a	83.00 ± 5.02 ^a
Glucose	–	168.42 ± 5.77	163.71 ± 6.48	165.85 ± 5.59	175.42 ± 4.54

Values are expressed as means ± SEM.

(a,b) Different characters indicate statistically significant differences in the same line (P < 0.05) for ALP and AST values.

(a,b) Different characters indicate statistically significant differences in the same line for ALT (P < 0.001).

One-way analysis of variance by Duncan test, P < 0.05, P < 0.001.

*ALP: alkaline phosphatase (IU/L), **AST: aspartate aminotransferase (IU/L), ***ALT: alanine Aminotransferase (IU/L); glucose (mg/dL); NGC: negative control group; group 1: control; group 2: CYP-treated rats; group 3: CYP + JWH-133-treated rats; group 4: JWH-133-treated rats.

Table 3. Oxidative stress parameters in male rats treated with CYP and/or JWH-133.

	NGC (n: 6)	Group 1 (n: 7)	Group 2 (n: 7)	Group 3 (n: 7)	Group 4 (n: 7)
SOD [•]	80.01 ± 5.35 ^b	84.03 ± 7.68 ^b	75.96 ± 1.31 ^b	51.77 ± 3.46 ^a	56.69 ± 5.53 ^a
MDA ^{**}	81.80 ± 5.07 ^{ab}	77.35 ± 6.93 ^{ab}	86.42 ± 7.21 ^b	81.69 ± 6.67 ^{ab}	61.43 ± 8.39 ^a
GPx ^{***}	71.84 ± 5.33 ^a	75.10 ± 1.26 ^a	50.07 ± 7.68 ^b	60.44 ± 4.59 ^{ab}	50.45 ± 3.21 ^b
SOD/GPx	1.13 ± 0.09 ^b	1.12 ± 0.10 ^b	1.75 ± 0.26 ^a	0.89 ± 0.10 ^b	1.13 ± 0.10 ^b

Values are expressed as means ± SEM. (a,b,c) Different characters indicate statistically significant differences in the same line (P < 0.05). SOD[•]: superoxide dismutase (U/g protein); MDA^{**}: malondialdehyde (µM/g protein); GPx^{***}: glutathione peroxidase (U/g protein). NGC: negative control group; group 1: control; group 2: CYP-treated rats; group 3: CYP + JWH-133-treated rats; group 4: JWH-133-treated rats.

(Figures 2b and 2c). The vessels were hyperemic. Remark cords had dissociative appearance and in places Kupffer cells were increased in number. Focal MCI and binucleated and giant-nucleated hepatocytes were observed in some places (arrow). The rats showed severe parenchymal degeneration (++++) and necrosis in the liver epithelium cells in group 3 (Figures 2d and 2e). In addition,

histopathological changes including perivascular round-cell infiltration, degeneration of hepatic cord, hyperemia in veins, an increase in the number of Kupffer cells, and giant multinucleated hepatocytes were noted. In group 4, veins were hyperemic, especially in the sinusoids close to the Glisson capsule, and mild parenchymal degeneration (+) was observed in the liver epithelial cells. An increase in

Table 4. Scoring of distribution of CB2 receptor expressed on liver as type of cells.

	NGC	Group 1	Group 2	Group 3	Group 4
Hepatocyte	-	-	-	-	-
Fibroblast	+	+	++++	++++	++
Biliary duct epithelium	+	+	+++	+++	+
Kupffer cell	-	-	+++	+++	+
Mast cell	-	-	+++	+++	-

Semiquantitative scoring of staining intensities for CB2 receptor of each group.

Semiquantitative scoring of immunostaining intensities: -, negative; +, very weak; ++, weak; +++, moderate; +++++, strong. NGC: negative control group; group 1: control; group 2: CYP-treated rats; group 3: CYP + JWH-133-treated rats; group 4: JWH-133-treated rats.

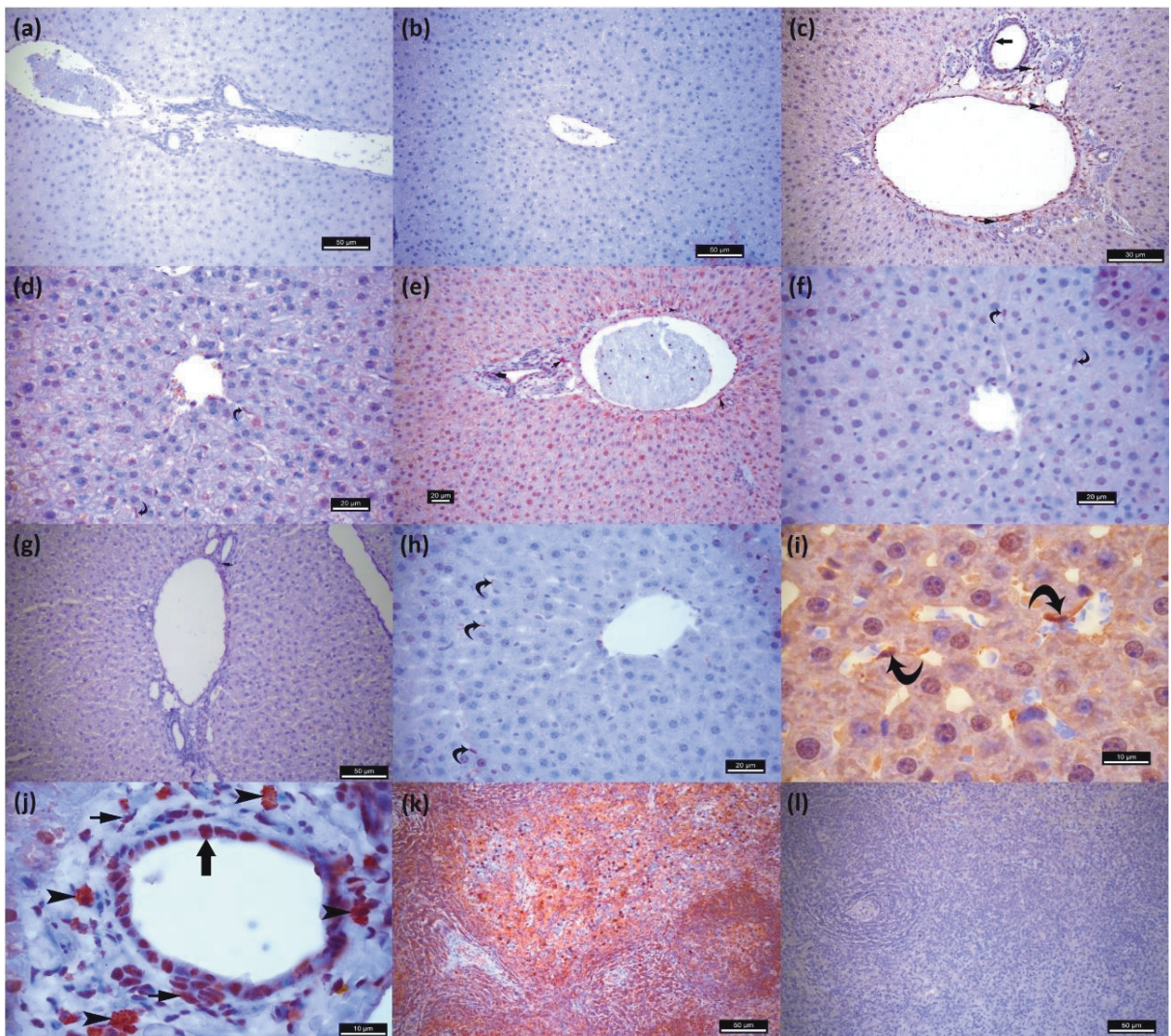


Figure 1. Representative distribution of CB2 receptor immunostaining on liver tissue sections prepared from control group (a, b), cypermethrin-treated groups (c, d, e, f, i, j) and just JWH-133-treated group (g, h). CB2 receptors were expressed in fibroblasts (thin arrow), bile duct epithelium (thick arrow), mast cells (arrow head) and Kupffer cells (curved arrow) in the liver. Positive control (k) and negative control (l) in rat spleen.

the Kupffer cell numbers and multinucleated hepatocytes (arrows) were observed in the liver (Figure 2f). It was observed that Kupffer cell numbers increased in groups 2, 3, and 4 as compared to the control group.

3.7. CB2 mRNA expression

The expression of CB2 in the liver was confirmed using QRT-PCR. Liver amplification of CB2 occurred after the 23rd cycle (Figure 3). There were no statistically significant differences in CB2 mRNA levels among the groups ($P > 0.05$).

4. Discussion

CYP, one of the safest pesticides used to kill a variety of pests, can cause toxic effects due to chronic exposure to nontarget mammals. CYP shows its toxic effects in different pathways. These pathways include modulation of the synthesis and release of neuromediators, oxidative stress, DNA damage, modulation of sodium, potassium, voltage-gated calcium, and chloride channels (3,4). The endocannabinoid system is responsible for various

activities such as pain, inflammation, appetite, and psychoactivity in mammals. The system creates these effects via various receptor- and nonreceptor-mediated pathways (22). A variety of natural or synthetic substances bind to endocannabinoid system receptors. Recent studies on organic phosphorus compounds have shown that these compounds act directly on fatty acid amide hydrolase and monoacylglycerol lipase responsible for the degradation of endocannabinoids (23). In one study, it has been indicated that WIN 55,212-2, CB agonist, modulates the effects of organic phosphorous insecticides (24). In other studies, CB2 activation has been emphasized to inhibit adenylate cyclase (25) and increase intracellular free Ca^{2+} levels (26). In the present study, JWH-133 increased the toxic effect of CYP on the liver. This may be due to the nonreceptor-mediated mechanisms mentioned above.

In our study, a decrease in weight gain was observed in the groups treated with CYP (groups 2 and 3) compared to the control group. It has been found that CYP reduced the weight gain in laboratory animals in similar studies

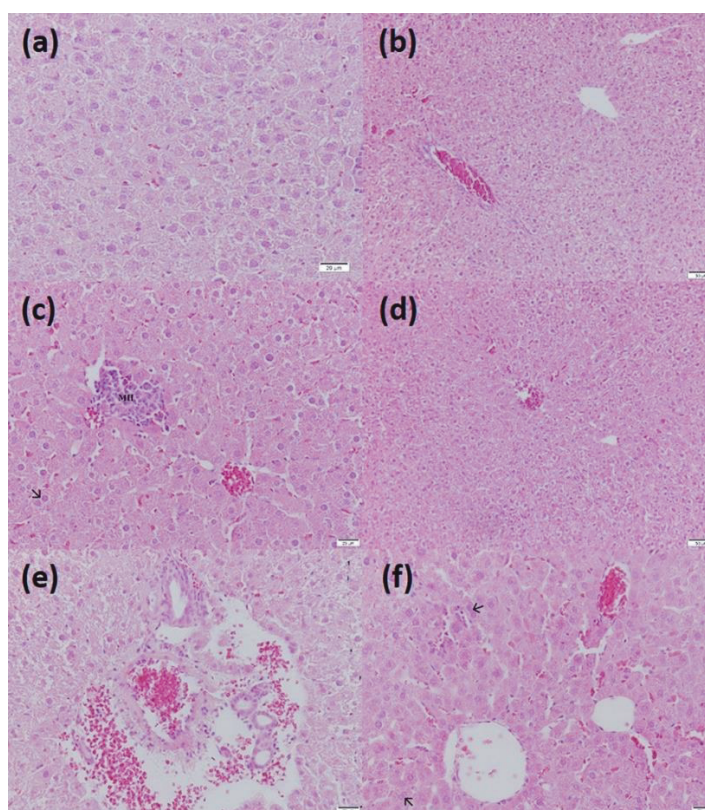


Figure 2. Light micrographs of rat livers treated as follows: control (a), cypermethrin-treated rats (b, c), cypermethrin plus JWH-133-treated rats (d, e), and JWH-133-treated rats (f). In group 2, moderate (++) parenchymal degeneration was observed. In group 3, severe (+++) parenchymal degeneration was observed. In group 4, mild (+) parenchymal degeneration was observed.

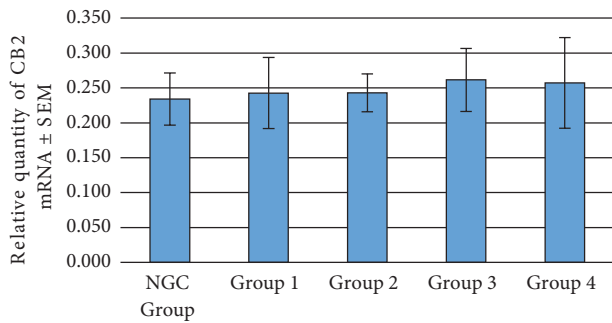


Figure 3. CB2 mRNA levels in groups. CB2 expression in the liver. Amplification in NGC and control group occurred after the 23rd cycle. There was no statistically significant increase in the level of CB2 mRNA in the groups. Data were calculated as the mean \pm SEM of the relative amount of mRNA. NGC: negative control group; group 1: control; group 2: CYP-treated rats; group 3: CYP + JWH-133-treated rats; group 4: JWH-133-treated rats.

(5,27,28). Group 3 had less weight gain among the groups. The reason for the slight increase in weight in group 3 may be the fact that JWH-133 increased toxic effects of CYP on rats. Clinical findings of sciatic nerve paralysis, salivation, timid behavior, drowsiness, itching, ataxia, convulsions, and hypersensitivity were observed in rats treated with CYP. These symptoms were also observed in other similar studies (5,28). Regarding the relative liver weight, Aldana et al. (5) reported a 20% increase in the 7-day CYP toxicity study. However, there were no significant changes in relative liver weights in a 28-day CYP toxicity study (6). In the present study, there were no significant differences in relative liver weight among the groups.

Lipid peroxidation plays a critical role in the pathogenesis of many human and animal diseases. In lipid peroxidation, free radicals such as hydroxyl and superoxide are formed and cause oxidative cell damage (29). Cellular damage leads to impaired liver function (8). Antioxidant enzymes are the first protection mechanisms used to protect biological macromolecules from oxidative damage (29). Studies have shown that oxidative tissue damage occurs in rats and mice exposed to pyrethroids (30,31). An increase in MDA level and a decrease in GPx and SOD activities in liver damage caused by CYP have been reported (7,8). These results are similar to results obtained from rats in group 2. CB2 agonists reduce MDA level in liver injury induced by I/R in mice (32,33). In the present study, JWH-133 did not significantly reduce the MDA level raised by CYP. Compared to the control group, there was a significant decrease in the level of MDA in group 4 ($P < 0.05$). Unexpectedly, significant decreases in SOD and GPx enzyme activities in groups 3 and 4 ($P < 0.05$) occurred. The depletion of antioxidant enzymes can be explained by the formation of reactive

oxygen species created by CYP, depletion of enzyme substrate, and/or downregulation of the transcription and translation process (34). Elevated superoxide production caused by lipid peroxidation was normalized by SOD and allopurinol or its metabolite oxypurinol, which are inhibitors of xanthine oxidase (generate superoxide) in the experimental diabetes studies in mice (35,36). In another study, JWH-133 reduced the formation of superoxide (37). These studies show that JWH-133 can reduce oxidative stress by reducing the amount of superoxide. In the present study, JWH-133 reduces the enzyme activity, and the level of MDA in this study can be explained in two ways: I) by reducing the formation of the superoxide by inhibition and/or downregulation of radical-producing oxidant enzymes (35,36); II) directly as antioxidants (Vit E, C, etc.) reducing the superoxide formed (31,38). As a result, antioxidant enzymes may have been downregulated because of the reduction in the level of superoxide by JWH-133. In some studies, researchers use the ratios between the activities of antioxidant enzymes (SOD/GPx) to examine oxidative stress (39,40). In the present study, the SOD/GPx ratio in group 2 was different from that of the other groups ($P < 0.05$). The lack of statistical significance ($P > 0.05$) among the other groups may be due to the oxidative stress-reducing effect of JWH-133.

It has been reported that agonists of CB2 have a protective effect against hepatic damage by reducing endothelial cell activation, inflammatory cytokines, activation of inflammatory cells, adhesion, and adhesion molecules (32,33). Thus, the antifibrogenic effect of CB2 activation on cirrhotic animals may be related to the antiinflammatory effect. The increased inflammatory response further increases oxidative stress and starts a bad cycle, eventually leading to cell death and organ dysfunction (33). In this study, according to the results, JWH-133 reduced lipid peroxidation in normal rats, but did not significantly reduce lipid peroxidation caused by CYP toxicity. The decrease in oxidative stress in group 3 may be due to antiinflammatory effects by CB2 activation.

In previous studies, it has been found that serums LDH, ALP, AST, and ALT activity increases in CYP-treated rats (5,7,8). Aldana et al. (5) reported that hepatic enzymes had risen until the 5th day and subsequently began to fall in rats given CYP for 7 days. In this study, the decrease in activity of serum ALP may be due to the development of resistance of the liver to CYP exposure. Serums AST and ALT activity in liver damage models was reduced by CB2 agonists (32,41,42). In the present study, CB2 agonist JWH-133 did not alleviate CYP-induced liver damage. In contrast, it enhanced the toxic effect of CYP on rats. The results obtained in this study are different from those of CCl_4 and I/R liver damage models. The differences between the results obtained in this study and

those obtained in CCl₄ and I/R liver damage models, as well as significant decreases and increases in enzyme activities in the mentioned studies, may be related to the differences emerging in effects and in effect mechanisms in comparison with damage models, and effects that are very quick and strong.

Data related to expression of CBs in liver are scarce. It has been previously indicated that normal liver does not express CB2 (43,44). In contrast, strong CB2 immunostaining was shown in human cirrhotic samples (43) and human samples with steatosis and nonalcoholic steatohepatitis (44). Expression of CB2 was increased and predominantly localized in the fibrous septa and portal tracts in the cirrhotic rat liver (45) and CCl₄-treated mice liver (42). In the present study, CB2 mRNA was detected in normal liver. There were no significant changes in CB2 mRNA expression levels among the groups. This may be explained by the presence of lymphocytes and macrophages that normally express the CB2 (44). In contrast, CB2 immunostaining was observed in the fibroblast cells, mast cells, Kupffer cells, bile duct epithelium, and intravenous leukocytes in CYP-treated liver. CB2 protein expression was very weak (+) in the control group. It was noted that there was no correlation between CB2 mRNA and protein expression in this study. When compared to similar studies, it has been shown that there is no correlation between mRNA levels and related protein levels in many genes (46–48). In addition, Schwanhäusser et al. (49,50) reported that proteins (approximately 46 h) were 5 times more stable than mRNA (9 h) in their work on mRNA and related protein half-life. Parallel to the results of these studies, Montecucco et al. (37) found a significant decrease of CB2 mRNA levels in mouse hearts after 30 min ischemia and 24 h reperfusion by comparison with hearts from sham-operated animals. Transcription, mRNA degradation, translation, and protein degradation are important processes that determine protein concentration. There was not a good agreement about the results between RT-PCR and IHC in the present study, maybe due to taking tissues 24 h after the last CYP treatment. The effect of CYP may be weakened and CB2 mRNA may be destroyed at this time. Since the degradation period of the CB2 protein is longer than CB2 mRNA, CB2-immunopositive staining may have been observed in IHC. In addition, there were no significant changes in CB2 mRNA expression among the groups, maybe due to the fact that CB2 did not express in hepatocytes, which are the primary cells of the liver; CB2 expressed in fibrogenic cells, which are very few in comparison with hepatocytes in the liver.

Histopathological examination of the present study revealed normal hepatic tissue histology in the control group. Alterations ranging from moderate degenerative (++) to necrotic were ascertained in group 2. Findings

observed in this study were in agreement with those observed in CYP toxicity studies (2,5,8,27). The role of CB2 in liver diseases is controversial. Some studies have indicated that CB2 activation shows protective properties during liver damage. The effects are usually based on the antifibrogenic or antiinflammatory effect induced by CB2 expressing liver fibrogenic cells and/or hepatic immune cells (32,33,43). Auguet et al. (51) reported that hepatic CB2 expression did not have significant differences between morbidly obese with normal liver, simple steatosis, or nonalcoholic steatohepatitis, and they also stated a positive correlation between liver CB2 and gene expression of adiponectin, which is an antiinflammatory factor. Authors have reported that CB2 is a molecule with a dual action. In another study, it has been demonstrated that CB2 potentiates obesity-associated fatty liver (52). In the present study, severe parenchymal degeneration (+++) and necrosis were observed in the rats in group 3. The pathologic condition was worse in rats treated with CYP and JWH-133 by comparison with rats treated with CYP only. Histopathological findings revealed a decrease in the number of immune cells in group 3 in comparison with group 2. Activation of CB2 may have an antiinflammatory effect. Taken together, JWH-133 increased CYP-induced liver toxicity in rats. Rats in group 4 showed very mild parenchyma degeneration (+) and hyperemic vessels (especially the sinusoids close to Glisson capsule) in liver. Interestingly, Kupffer cell proliferation was observed in group 4 compared to the control. Deveaux et al. (52) reported that the infiltration of adipose tissue macrophages was increased by JWH-133 in inflammation at an early stage and decreased by the inhibition of CB2 in obesity. In the present study, the increase in Kupffer cells may be due to hepatotoxicity induced by CYP in groups 2 and 3, and may be due to JWH-133 in group 4.

Mast cells have an important role in liver disease processes such as cirrhosis, fibrosis, hepatitis, and regeneration after liver injury. In both cases, the number of mast cells usually increases (53–55). CB2 mRNA was found in mast cells (56,57). The role of mast cells in liver I/R injury has not been fully understood. In one study, it has been reported that mast cells do not play a role in hepatic I/R injury. However, mast cells may not be seen because this study was performed *in vitro*, and the data were collected 1 h after reperfusion (58). In another study, it was found that mast cells had an important role in I/R injury, and degranulation of mast cells increased in this injury model (59). CB2 activation has many different effects on mast cells. JWH-133 reduced edema induced by mast cells (60). HU-308, which is a CB2 agonist, reduced arachidonic acid in ear edema (61). However, application of CB1 and CB2 agonists and antagonists to rat peritoneal mast cells did not affect histamine release (62). In another study,

2-arachidonoylglycerol and CP55,940 decreased mast cell activation by being susceptible to a CB2 antagonist and inhibition of nitric oxide and prostanoid pathways (63). In the present study, CB2 expression was not observed in groups 1 and 4, but it was observed in groups treated with CYP. There is little information about the mechanisms and internal molecules that have the ability to modulate excessive mast cell activity. Taken together, like the Kupffer cell, the mast cells may also have a role in the pathophysiology of hepatic damage induced by CYP.

The increase in CB2 expression in some cells, histopathological findings, and changes in hepatic enzyme activities in liver damage induced by CYP showed that JWH-133 enhances the toxic effect of CYP on rat liver. JWH-133 reduced oxidative stress but did not reduce liver

damage. Another important consequence of this study is the increased CB2 density in the liver in the case of CYP toxicity. Our results suggest that CB2 (receptor mediated or nonreceptor-mediated) might be involved in the physiopathological process of CYP-induced liver injury and justify the need for further study. Because the results of this study contradict the results of studies on CB2, the results should be confirmed by further studies to be carried out in more detail.

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