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The immunological and histopathological changes of Tramadol, Tramadol/Acetaminophen and Acetaminophen in male Albino rats "Comparative study"

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Abstract

Background:

Tramadol is a synthetic opioid analgesic. It is commonly prescribed for moderate to severe pain, becoming abused more popular among teens in most countries. Paracetamol as anti-inflammatory drugs (acetaminophen) (APAP) is widely used as an analgesic and antipyretic agent. Meanwhile, tramadol/acetaminophen (tramacet) is effective in acute or chronic moderate-to-moderately severe pain. In comparative study, the current investigation threw the light on the effect of over doses of tramadol and/or APAP on the immune function and hepatocytes in adult male Sprague-Dawley rats.

Material and methods:

Treated rats received oral doses of each drug for 15 consecutive days and after last treatment, they kept three days later for withdrawal studies. The rats were divided into four treatment groups, in the first group, rats received saline and used as control. The second, third and fourth groups treated with tramadol (45 mg/kg), tramadol/APAP (45/450 mg/kg), APAP (450 mg/kg) respectively, once a-day at the first week and ending with 90, 90/900, 900 mg/kg at the second week. Rats were sacrificed at the end of the first, second weeks and three days of last treatment.

Results:

Daily doses of tramadol and /or APAP exposure in rats decreased the cellularity of spleen. Moreover, phagocytic and killing of *S. aureus* by PMN and macrophage cells caused a highly significant decrease in treated groups. IFN-γ was reduced in a statistically different treated group of rats. Serum IL-10 was unaffected by any of the treatment regimens but increased only in tramadol/APAP treated rats. Spleen histology exhibited mild pathological alteration with different injures between treated groups. Splenic white pulp accompanied by ill deformed which reflected the reduction of white pulp zones, thickened vasculature in the splenic net work, fibrous trabeculae become prominent feature, where splenic red pulp occupied large areas of the splenic network with predominant edema and megakaryocytes. On the other hand, the effect of tramadol and/or APAP induced DNA alterations of hepatocytes in dose dependent pattern as elucidated by dendrogramatic analysis. Liver histopathological changes of treated groups included vacuolated hepatocytes, dilated sinusoid with proliferated Kupffer cells; atrophied hepatocytes with nuclei reduced in size and darkly stained. Many areas of hepatocytes showed loss of architecture, congested central vein, expanded portal area with edema and inflammatory reaction.

Conclusion:

It could be concluded that the effect of tramadol/APAP induced anti-inflammatory cytokines than tramadol and APAP alone. Tramadol and/or APAP may display severe pathological consequences of hepatocytes. These hepatic lesions may be caused impairment of the liver function.

Keywords: Tramadol, Tramadol/Acetaminophen, Acetaminophen, Cytokines, Phagocytosis, Histopathology of spleen and liver, DNA fragmentation, Albino rat.

Introduction:

The nature of the immune-modulatory activity of the opioids has been the subject of a great deal of research for the last years. There is increasing evidence that effects of opioids on the immune response are mediated at several levels. Modulation of the inflammatory response appears to be a target of these compounds, including effects on phagocytic activity, as well as, the response of cells to various chemo-attractant molecules. Moreover, the findings from several laboratories have demonstrated the impact of opioid treatment on antibody responses, and the molecular basis for this effect is likely due, at least in part, to the modulation of both cytokine and cytokine receptor expression (McCarthy *et al.,* 2001; Liu *et al.,* 2008).

Tramadol is a synthetic opioid analgesic commonly prescribed for moderate to severe pain, usual doses being up to 200 mg/day (Gana *et al*., 2006; McKeon *et al.,* 2011). The maximum allowed daily dose is 400 mg. Tramadol provides analgesia through 3 mechanisms: mu-opioid binding (through its metabolite O-desmethyltramadol), serotonin reuptake inhibition (through (+) -tramadol) and nor-epinephrine reuptake inhibition (through (-)-tramadol).O-desmethyltramadol (which is formed from tramadol through O-demethylation catalyzed by CYP2D6) is responsible for the opiate-type effects of tramadol (Reeves and Burke, 2008; Sansone and Sansone, 2009). Tramadol is also thought to have some NMDA-type antagonist effects, which has given it a potential application in neuropathic pain states (Lintz *et al.,* 1998). Increasing serotonin and norepinephrine may also reduce inflammatory cytokines which are released by the brain in response to stress. The inflammatory cytokines would have slowed down recovery from a workout or illness - impairing one's immune system and healing, thus tramadol may have an anti-inflammatory effect (Bianchi *et al.,* 1999; Barkin, 2008).

Tramadol abuse is becoming more and more popular among teens in most Countries (including US). Being an opioid, tramadol carries all possible risks known from other opiates (Cicero *et al*., 2005; Adams *et al*., 2006). Side effects include dizziness, headache, somnolence, nausea, constipation, sweating, pruritus, and central nervous system stimulation (Reig, 2002; Kabel and van Puijenbroek, 2005). Tramadol causes respiratory depression, although usually weaker than that seen with other opiates and opioids (Senay *et al*., 2003). Tramadol can cause psychological and physical addiction similar to that of other opiates and the analgesic efficacy of tramadol can further be improved by combination with a non-opioid analgesic (Ripamontic *et al*., 2004; Lanier *et al.,* 2010).

The young addicts in our population typically substituted tramadol for heroin. Repeated tramadol administration in such patients might lead to the accumulation of toxic metabolites in the body, increase the risk for pharmacokinetic interactions, and/or decrease the clearance of tramadol, thus increasing its potential for toxicity (Tjäderborn *et al.,* 2007; De Decker *et al.,* 2008; Shadnia *et al.,* 2008). Tramadol is associated with the development of a physical dependence and a severe withdrawal syndrome (Brinker *et al.,* 2002; Barsotti *et al.,* 2003). Tramadol causes typical opiate-like withdrawal symptoms as well as atypical withdrawal symptoms including seizures. The atypical withdrawal effects are probably related to tramadol's effect on serotonin and norepinephrin reuptake.

Paracetamol as anti-inflammatory drugs (acetaminophen) (APAP) is widely used as an analgesic and antipyretic agent, with an excellent safety profile within the therapeutic dose range (up to 4 g/day) (Lipton *et al.,* 2000; Prior *et al.,* 2002). The main safety concern with using paracetamol is to avoid over dosage; the intake of an overdose of APAP frequently causes severe acute liver injury (Whitcomb 1994; Makin and Williams 1997). APAP is metabolized by cytochrome P450 to generate a toxic metabolite,N-acetyl-p-benzoquinone imine (NAPQI), which can reduce glutathione (GSH) in the liver (Dahlin *et al.,* 1984). Thus, an overdose of APAP depletes hepatic GSH, and NAPQI covalently binds

to cysteine residues on proteins, resulting in the formation of 3-(cysteine-S-yl) APAP adducts, which lead to liver injury (Ishida *et al.,* 2004; Stannard and Booth, 2004).

Tramadol/APAP (tramacet) is effective in acute or chronic moderate - to -moderately severe pain (Schnitzer, 2003). Its benefit from the complementary actions of the constituent analgesics, having the rapid onset of APAP and the sustained effect of tramadol (Dhillon, 2010). The main analgesic options that are currently available include non-steroidal anti-inflammatory drugs (NSAIDs; both traditional non-selective and cyclo-oxygenase (COX)-2 selective agents) paracetamol and opioids (tramacet) (Raffa, 2006; Cavazzini, 2008). A single dose pharmacokinetic study of tramacet in volunteers showed no drug interactions between tramadol and APAP. Upon multiple oral dosing to steady state, however, the bioavailability of tramadol and metabolite M1 (O-desmethyl-tramadol) was lower for the combination tablets compared to tramadol administered alone. The cause of this reduced bioavailability is not clear. Following single or multiple dose administration of tramacet, no significant change in APAP pharmacokinetics was observed when compared to APAP given alone (Fricke *et al.,* 2004; Bourne *et al.,* 2005). The clinical presentation of overdose may be included the signs and symptoms of tramadol toxicity, APAP toxicity or both.

In a previous investigation Mostafa (2006) demonstrated that tramadol produced a slight decrease in the cellularity of the spleen of rats during acute, chronic and withdrawal periods. It produced a slight decrease of ConA-stimulated proliferation of T-cells and LPS-stimulated proliferation of B-cells. Production of IFN-γ by splenic cells was significantly impaired during tramadol treatment and withdrawal. The results showed also that the levels of IL-6 were decreased at dose 12mg/100g and significantly increased at dose of 8mg/100g. Histopathological changes in spleen were detected after tramadol administration in a dose dependence manner.

IFN-γ exerts pleiotropic effects including antiviral and bactericidal activities, activation of macrophages, NK cells, and up-regulation of MHC class II expression on macrophages. It is produced mainly by NK cells and Th1 cells (Farrar and Schreiber, 1993) and has been reported to be involved in various kinds of liver injury models (Mizuhara *et al.,* 1996; Mihm *et al.,* 1996). Enhanced IFN-γ expression is presumed to induce inflammatory responses, leading to parenchymal cell damage in the liver. In APAP-induced liver injury, IFN-γ levels in the liver were correlated with severity. Clinically, APAP hepatotoxicity was markedly enhanced in patients treated with IFN-γ (Kellokumpu-Lethinen *et al.,* 1989). In spite of the beneficial role of these anti-inflammatory drugs, these drugs incriminated in some body organs (Harrill and Rusyn, 2008).

From all previous studies, there are a few studies on immune function. In addition, we did not find any Egyptian studies deal with the immunological and cytological finding associated with tramadol/APAP. Therefore, the present study is interest to investigate the immunomodulatory effects and the toxicity of tramadol, tramadol/APAP and APAP in comparative study when administered after two weeks followed by withdrawal period on the immune function in adult albino rats. In addition, evaluating the inductive effect of these drugs on histological changes in the liver and spleen tissues were investigated.

MATERIAL AND METHODS

Animals

Male albino rats of the Sprague-Dawley, 60 days old and 150 to 180 g in weight were used as experimental animals throughout the present work. They were supplied from Theodore Billharz Research Institute (TBRI). Upon arrival, the rats were individually caged in a colony room, where a reversed day-night (12 hr) cycle was maintained through artificial illumination. Rats received access free to both food pellets (protein 21% and energy 2950 K.C.) and water throughout the experiments.

Drugs:

Tramal : Tramadol hydrochloride tablets produced by Global Napi Pharmaceuticals, Egypt.

Tramacet: Tramadol hydrochloride and APAP combination tablets produced by Delta Pharma, S. A. E., Tenth of Ramadan City, A. R. E.

Tylenol : Acetaminophen tablets (APAP) distributed by McNeil Consumer Health Care, Division of McNeil-PPC. INC, Fort Washington, PA 19034 USA.

Experimental design

The animals (84 rats) were randomly assigned to four treatment groups (n=21), in first group, rats received oral dose (PO.) of 0.1 ml saline for the same period of experiment, and used as control. The second, third and fourth groups as treated. Treated rats received oral doses of each drug for two weeks, after last treatment, animal treated were kept three days later for withdrawal studies. In the 2nd group, rats received daily oral doses of tramadol Hcl with increasing this dose weekly started with 45 mg/kg/day at the first week and ending with 90 mg/kg/day at the second week, In the 3rd group, treated rats received daily treatment with tramadol/APAP tablets with increasing dose weekly, starting with 45/450 mg/kg/day at the first week and ending with 90/900 mg/kg/day at the second week, The 4th group, treated rats administered daily oral doses of APAP with increasing dose weekly, starting with 450 mg/kg/day at the first week and ending with 900 mg/kg/day at the second week. Treated rats then kept three days later to study withdrawal symptoms. Seven animals from each group were scarified at the end of the first, the second week of treatment and after three days later of last administration of different drugs. These daily doses were calculated for rats according to Paget and Barnes (1964) conversion tables.

Measurement of serum IFN-γ and IL-10

To investigate the effect of tramadol and/or APAP administration on T-helper (Th) cell population of lymphocytes [Th1 cytokine (IFN-γ)/Th2 cytokine (IL-10)] cell function and on this response, immunoassay kits {Abnova (cat # KA0274. V. 01), PBL Biomedical Laboratories (product # 43500-1, V.1.3)} were used to determine serum concentration of IFN-γ (Kelder *et al.*, 1986) and IL- 10 (Moore *et al*., 1993) as an indicator of Th1 and Th2 cell function, respectively.

Phagocytosis and killing assay

Blood collected in heparin-coated universal bottles from experimental animals were used to prepare leukocytes for bacterial phagocytosis and killing. The heparinized blood samples were treated with 0.83% ammonium chloride to lyses red blood cells, washed three times with cold phosphate buffer solution (PBS) at PH 7.2 and resuspended in minimal essential medium (MEM) with 0.5% inactivated fetal calf serum to give a final concentration of 10^8 viable poly-morphonuclear leukocytes (PMN) and macrophage cells per ml (Woldehiwet and Rowan, 1990). A strain of coagulase-positive *Staphylococcus aureus* (*S. aureus*) isolated in the laboratory was used. The bacteria were washed in PBS and resuspended in (MEM) to give a final concentration of $5x10^8$ bacteria per ml.

Assay of phagocytosis

The mixtures of bacteria (*S. aureus*) and leukocytes were incubated at 37ºC for two hours with regular stirring and then the mixtures were centrifuged at 200xg for 5 min. at 4 ºC. The supernatants were used to estimate the percentage of bacteria phagocytosed using the formula: Phagocytosis % = (CFU before incubation - CFU after incubation) / CFU before incubation

Assay of bacterial killing

Samples of MEM containing bacteria (*S. aureus*) alone and containing

mixtures of bacteria (*S. aureus*) and leukocytes were incubated at 37ºC for two hours. Then the samples were treated with one cycle of freezing with liquid nitrogen and thawing. The number of colony forming units (CFU) was then estimated by the method of Woldehiwet and Rowan (1990). The percentage of bacteria killed was estimated according to the formula:
Bacterial killing % = CFU in sample containing bacteria - CFU in sample containing bacteria & leukocyte mixture /CFU in sample containing bacteria alone

Splenocytes preparation

The spleen of rats were individually removed and placed in cold culture media (RPMI-1640) containing 100U/mlpenicillin,100µg/ml,streptomycin, 200 mM Glutamine/100 mM Na pyruvate. Each spleen was gently teased loose and passed through a stainless steel mesh (40µm pores) to remove cell aggregates and connective tissue. Each suspension was centrifuged at + 4ºC for 10 min at 400xg. To lyses the RBCs, the pellet was resuspended in 1ml sterile dist. water (hypotonic solution) for 30 sec, then 40 ml of RPMI medium were immediately added and the suspension was centrifuged at+4ºC for 5 min at 400xg. Cells were then washed twice with ice-cold RPMI-1640 and resuspended for counting. Counting and assessment of viability were performed by trypan blue exclusion method (Bayer *et al*., 1990).

Qualitative analysis of DNA fragmentation of rat hepatocytes by agarose gel electrophoresis

The present protocol provides a method for DNA separation of fragmented intact DNA fractions for the analysis by garose gel electrophoresis. As described by Bortner *et al.* (1995).

The Cluster analysis

The percentage of each peak of the control and treated cells were analyzed using the cluster analysis system (AAB-Clustering analysis software, USA). The results were calculated on the bases of similarity coefficient values and average linkage which displays the homogeneity and heterogeneity as determined according to the simple band match (SBM) and amount of damage in the percentage in dendrogram.

Histopathological examination

At the end of each experimental period, all animals were sacrificed by cervical dislocation and their livers and spleens were isolated and rinsed in phosphate-buffered (pH 7.5), fixed in phosphate-buffered formalin, sections were prepared and stained with hematoxylin and eosin (H&E) according to Bancroft and Gamble, (2002).

Statistical analysis

Data were presented as mean ± SE. The statistically significant differences of treated and control animals were evaluated by Student's *t*-test or one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Effects of cellularity of spleen

The effects of daily tramadol and /or APAP exposure for 7, 15 consecutive days and withdrawal period on cellularity of spleen are presented in Fig. (1). Daily doses of tramadol and /or APAP exposure in rats for 7, 15 consecutive days and withdrawal period significantly decreased the cellularity of spleen of treated groups compared to the control group.

Effects of phagocytosis and killing of *S. aureus* by polymorphonuclear and macrophage cells in the peripheral blood

The effect of tramadol and/or APAP administrated for 7, 15 consecutive days and withdrawal period on phagocytosis and killing of *S. aureus* by PMN and macrophage cells in the peripheral blood are shown in Figs. (2 , 3). Tramadol and /or APAP exposure caused a highly significant decrease on phagocytic and killing of *S. aureus* by PMN and macrophage cells versus control group.

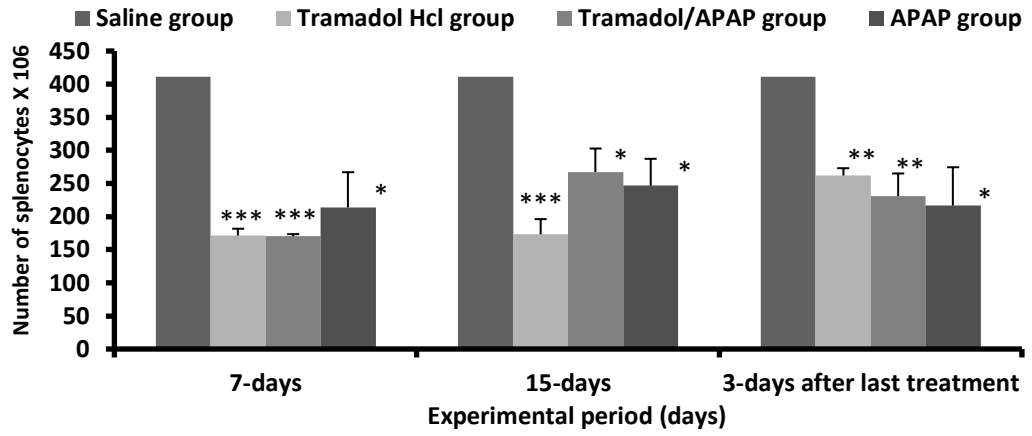

Fig.(1):Total number of splenocytes after different treatment groups in rats.
The data are expressed as mean ± S .E. * : $P \leq 0.05$, ** : $P \leq 0.01$, ***: $P \leq 0.001$

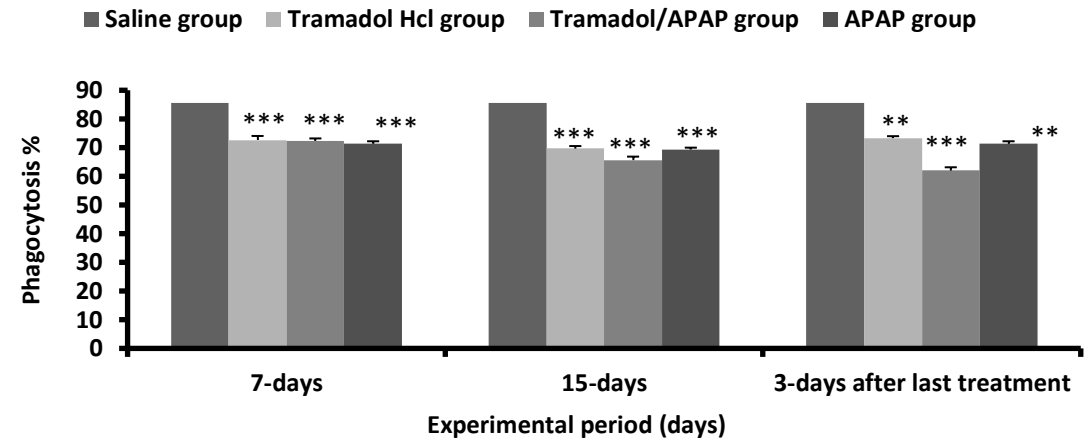

Fig.(2): The percentage of phagocytic bacteria by the polymorphonuclear leukocytes and macrophage cells in the peripheral blood after different treatment groups in rats.
The data are expressed as mean ±S.E . ** :$P \leq 0.01$, ***: $P \leq 0.001$

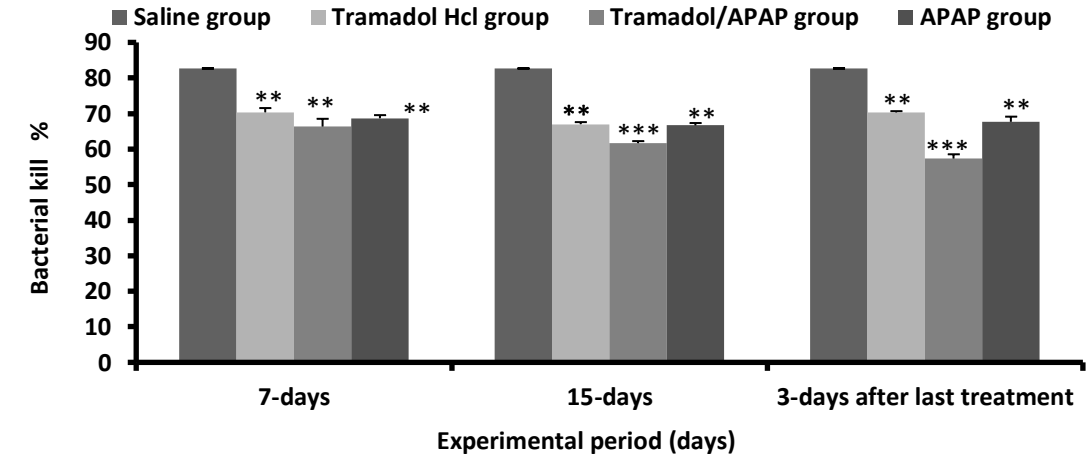

Fig.(3): The percentage of bacteria killed by the polymorphonuclear leukocytes and macrophage cells in the peripheral blood after different treatment groups in rats.
The data are expressed as mean ± S.E. ** : $P \leq 0.01$, ***: $P \leq 0.001$

Effects on serum IFN-γ and IL-10

The effects of daily tramadol and/or APAP administrated for 7, 15 days and withdrawal period on serum IFN-γ and IL-10 concentration are presented in Figs. (4, 5). The level of the Th1- related cytokine, serum IFN-γ was reduced in a statistically different treated group of rats versus the control group. On other hand, serum IL-10 concentration, Th2- related cytokine was unaffected by any of the treatment regimens except tramadol/APAP combination treated rats for 7 days which showed an increase in serum IL-10 concentration compared to the control group.

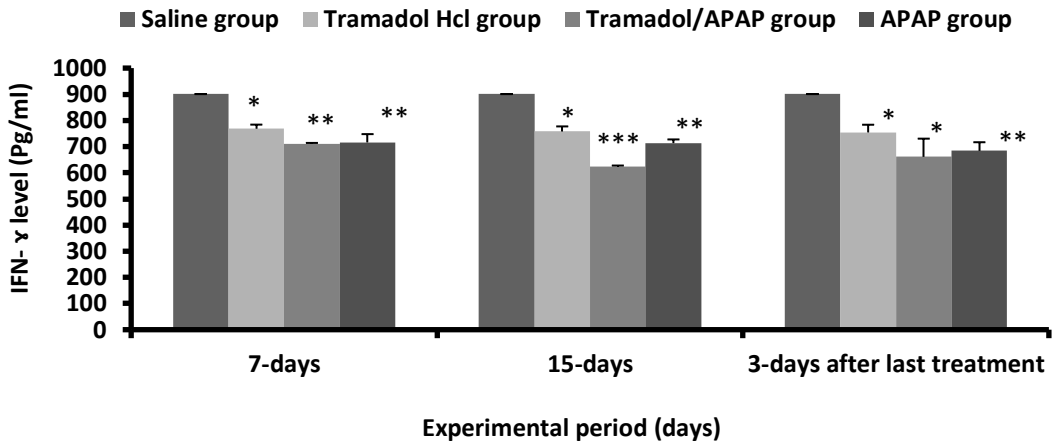

Fig. (4): Level of IFN- ɤ in serum after different treatment groups in rats.
The data are expressed as mean ± S. E . *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$

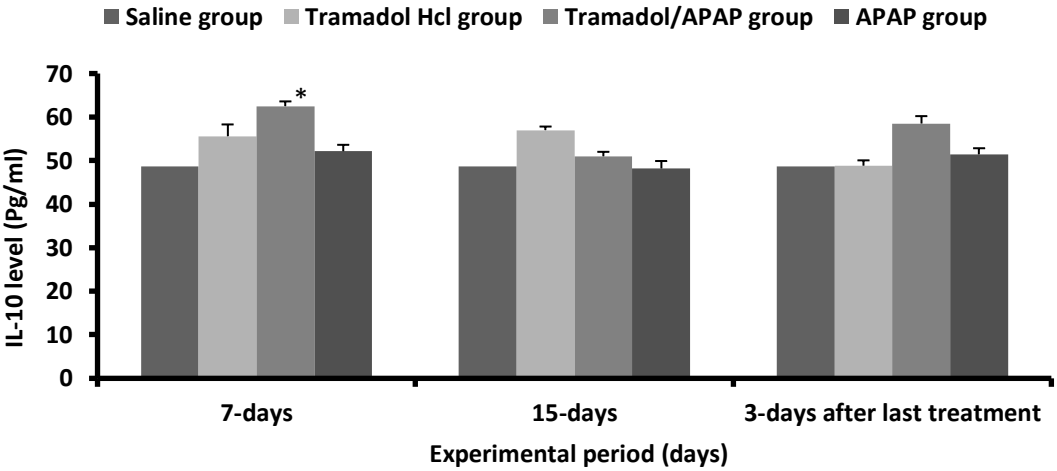

Fig. (5):Level of IL-10 in serum after different treatment groups in rats.
The data are expressed as mean ± S. E . *: $P \leq 0.05$

Histological finding:

Histological examination of spleen

Splenic tissues examination of daily tramadol and/or APAP administrated for 7 consecutive days showed similarity microscopic changes versus control group (Fig. 6). It exhibited mild pathological alteration, where, partial loss of splenic architecture was seen. Splenic white pulp accompanied by ill deformed, the demarcation between it and red pulp become more lost, thick central arteriole could be seen. Megakaryocytes and congested sinuses showed in the net work of the spleen sections (Figs.7, 8, 9, 10, 11).

The pathological profile of spleen sections of daily tramadol and /or APAP of treated group for 15 consecutive days showed thickened vasculature in the splenic net work, fibrous trabeculae become prominent feature, this beside increase in megakaryocytes incidence and mitotic activity could be seen in some white pulp lymphocytes reflecting the spleen activity (Figs. 12, 13, 14).

Histologic of splenic examination of tramadol and /or APAP of treated group after withdrawal period showed ameliorate to mild degree the pathological alteration, where splenic red pulp occupied large areas of the splenic net work, predominant edema and megakaryocytes could be seen in the red pulp and which reflected the reduction of white pulp zones (Figs. 15,16,17).

Histological examination of liver

Liver sections of daily tramadol and /or APAP administrated for 7 consecutive days showed similarity microscopic changes versus control group (Fig. 18). It displayed mild lose of liver architecture, most of vasculature were intact. However, few central veins of liver tissue showed dilation or congestion with lacerated (Figs. 19, 21, 23). Vacuolated hepatocytes, dilated sinusoid with proliferated Kupffer cells, atrophied hepatocytes with nuclei reduced in size and darkly stained could be seen. Mild perivascular inflammatory response was detected also in liver sections (Figs. 20, 22, 24).

The examination of liver tissue of rats treated with tramadol and/or APAP for 15 consecutive days revealed the same observation as in one week of treatment, in addition to increase the losing of architecture of liver tissue, while, inter lobular hemorrhagic areas and atrophied hepatocytes with eosinophilic homogenous cytoplasm, dilated sinusoid with inflammatory cells occasionally seen (Figs. 25, 28, 29, 31). Meanwhile, many areas of hepatocytes showed loss of architecture, congested central vein, expanded portal area with edema and inflammatory reaction (Figs. 26, 27, 30).

The liver of rats treated with tramadol and/or or APAP after 3-days of last treatment revealed moderate degree of improvement in hepatocytes, where, many blood vessels were normal, reduced inflammatory reaction, but, hepatocytes displayed with necrotic areas and lymphocytes in sinusoids and restricted portal area could be seen (Figs. 32, 33, 34).

Effects of DNA fragmentation of rat hepatocytes

The obtained results revealed that tramadol and /or APAP exerted DNA changes in different regime of applications. Briefly, Fig. (35) and Table (1) were represented DNA finger printing pattern obtained by DNA fragmentation by using agarose gel electrophoresis of rat hepatocytes. The results displayed that 1st group (control group) showed one band 1804 base pair (bp). The 2nd group after 1st week of tramadol treatment showed one band 1226.9 bp. While, the 3rdgroup of tramadol/APAP combination treatment displayed one band 1190 bp. Meanwhile, the 4th group of APAP treatment showed 3 bands ranging from 1261.6 to 844.3 bp. After 2nd week of the 2nd group of tramadol treatment showed four bands ranging from 1729.6 to 786.76 bp. While, the 3rd group of tramadol/APAP combination treatment displayed 3 bands ranging from 1729.6 to 773.73 bp. Meanwhile, the 4th group of APAP treatment showed 3 bands ranging from 1729.6 to 800 bp. After three days of last treatment, the 2nd group of tramadol

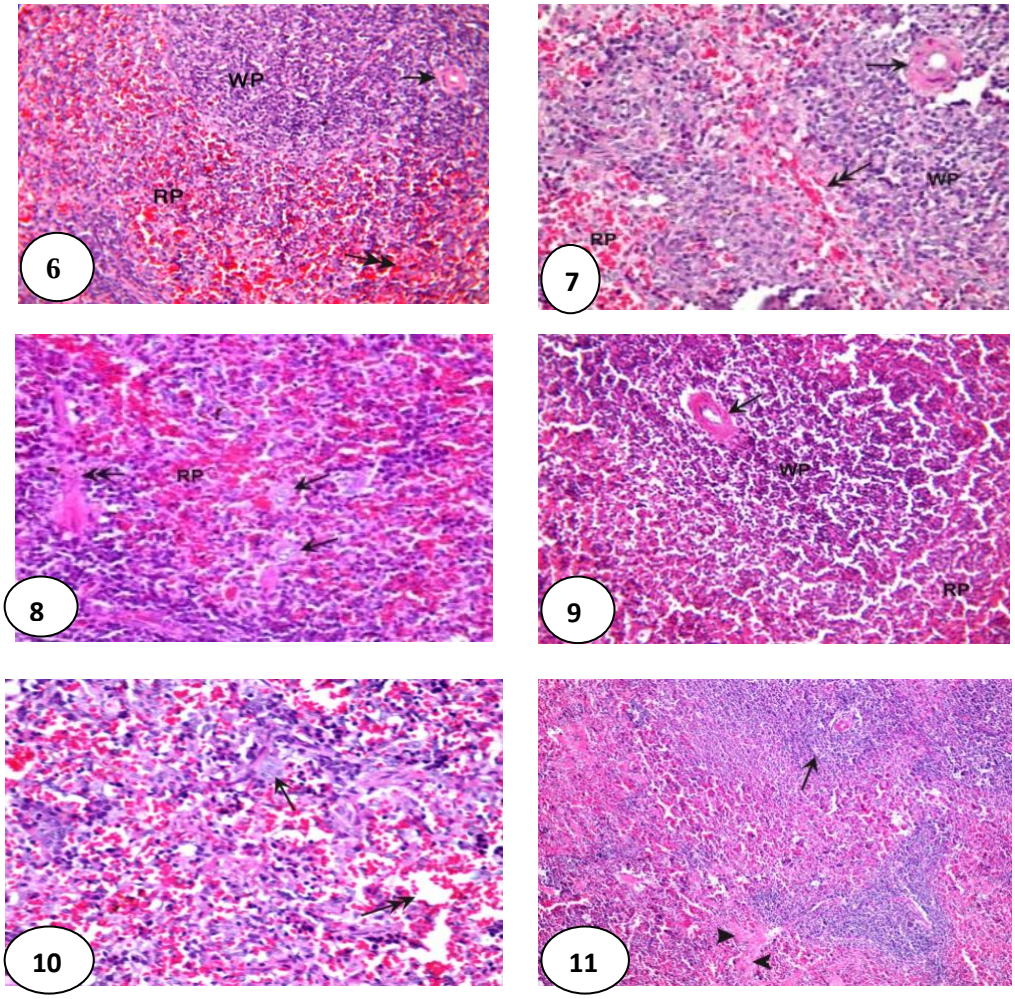

Fig. (6): Spleen section from the 1st group (control), showing normal structure of white pulp, central arteriole (arrow) and red pulp (double arrow), (H&E, X:100). **Fig. (7):** Photomicrograph of spleen tissue of the 2nd group after one week of oral tramadol treatment demonstrating diffuse white pulp (WP), thick central arteriole (arrow) and red pulp (RP), (H&E, X:100). **Fig. (8):** Spleen section from 2nd group after one week of oral tramadol treatment showing megakaryocyte cell (arrow) in red pulp (RP), thickened trabeculae (double arrow), (H&E, X:100). **Fig. (9):** Photomicrograph of spleen tissue of the 3rd group after one week of oral tramadol/APAP combination treatment showing, white pulp (WP) with ill deformed, no demarcation between white and red pulp (RP) and thick central arteriole (arrow), (H&E, X:100). **Fig. (10):** Photomicrograph of spleen tissue of 4th group after one week of oral tramadol treatment, demonstrating megakaryocyte cell (arrow) and dilated congested sinuses (double arrow) in the red pulp. (H&E, X: 100). **Fig. (11):** Spleen section from the 4th group after one week of oral APAP treatment, revealing reduction in some white pulp size (arrow), thick trabeculae (arrow head) in the red pulp.(H&E, X:40).

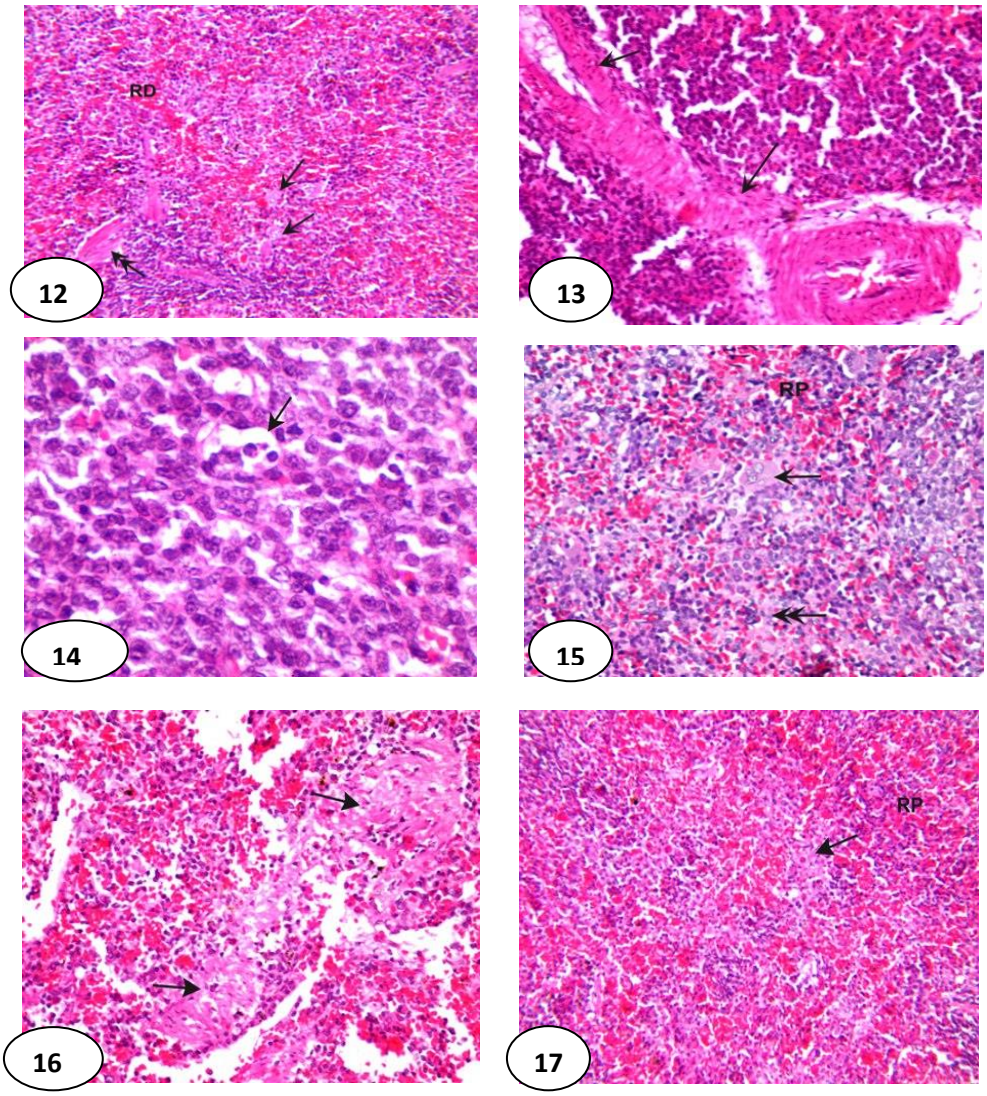

Fig. (12): Spleen section from the 2nd group after two weeks of oral tramadol treatment demonstrating megakaryocyte cell (arrow) and thickened trabeculae (double arrow) in the red pulp (RP). (H&E, X: 100). **Fig. (13):** Photomicrograph of spleen tissue of the 3rd group after two weeks of oral tramadol/APAP treatment showing thickened vasculature in the splenic net work (arrow) (H&E, X:100). **Fig. (14):** Spleen section from the 4th group after two weeks of oral APAP treatment showing mitotic activity in some lymphocytes within the white pulp, reflecting the spleen activity (arrow). (H&E, X: 200). **Fig. (15):** Photomicrograph of spleen tissue of the 2nd group (tramadol treatment) after three days of last treatment showing megakaryocyte cell (arrow) and edema (double arrow) in the red pulp (RP). (H&E, X: 100). **Fig. (16):** Spleen section from the 3rd group (tramadol/APAP combination treatment) after withdrawal period, demonstrating red pulp with edema in trabecula (arrow). (H&E, X: 100). **Fig. (17):** Photomicrograph of spleen tissue of the 4th group (APAP treatment) after withdrawal period, demonstrating edema predominant with red pulp, (arrow). (H&E, X: 40)

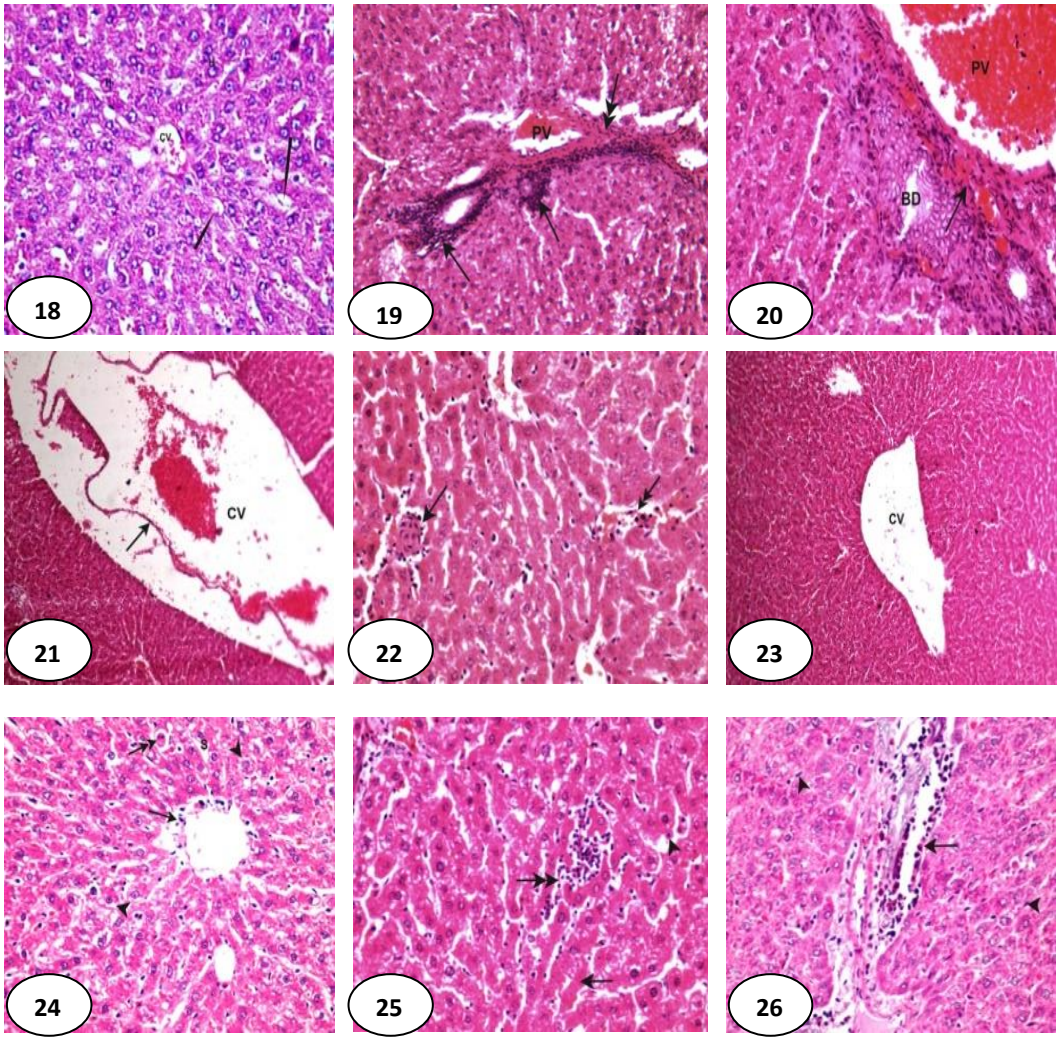

Fig. (18): Photomicrograph of liver tissue of the 1st group (control), showing central vein (CV) and intact hepatocytes (H&E, X: 200). **Fig. (19):** Liver section from the 2nd group for one week of oral tramadol treatment displaying expanded portal area, periportal inflammatory aggregates (arrow), fibrosis (double arrow) and portal vein (PV), (H&E, X:100). **Fig. (20):** Photomicrograph of liver tissue of the 2ndgroup after one week of oral tramadol treatment showing congested portal vein (PV) and fibrosis (arrow) bile duct (BD), (H&E, X: 200).
Fig. (21): Liver section from the 3rd group after one week of oral tramadol/APAP treatment displaying congested dilated central vein (CV) with lacerated wall (arrow), (H&E, X: 40). **Fig. (22):** Photomicrograph of liver tissue of 3rd group after one week of oral tramadol/APAP treatment showing necrotic area (arrow) and sinusoid with lymphocytic aggregates (double arrow), (H&E, X:100). **Fig. (23):** Liver section from the 4th group after one week of treatment, showing dilated central vein (CV), (H&E, X: 40). **Fig. (24):** Photomicrograph of liver tissue of the 4th group after one week of oral APAP treatment demonstrating mild pervascular inflammatory reaction (arrow), vacuolated hepatocytes (arrow head), dilated sinusoid (arrow) and hyaline body (double arrow), (H&E, X: 200). **Fig. (25)**; Liver section from the 2nd group after two weeks of oral tramadol treatment demonstrating hepatocytes with eosinophilic homogenous cytoplasm (arrow), dilated sinusoid with inflammatory cells, (double arrow) and hyaline body (arrow head), (H&E, X: 200). **Fig. (26):** Photomicrograph of liver tissue of the 2nd group after two weeks of oral tramadol treatment showing periportal lymphocytic infiltrate (arrow), von Kűpffer cells (arrow head), (H&E, X: 200)

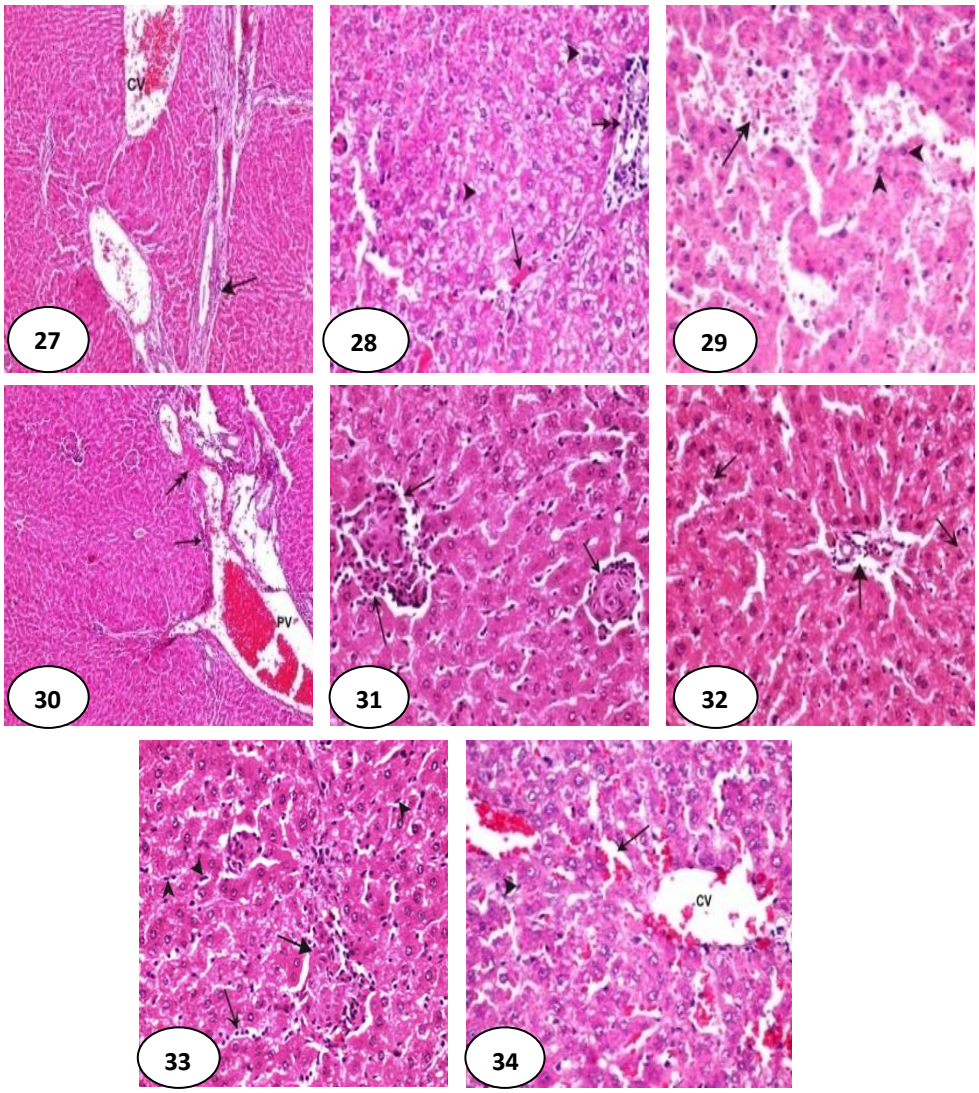

Fig. (27): Liver section from the 3rd group after two weeks of oral tramadol/APAP treatment showing dilated congested central veins (CV), perivascular inflammation in portal area (arrow), (H&E, X: 40). **Fig. (28):** Photomicrograph of liver tissue of the 3rd group after two weeks of oral tramadol/APAP treatment demonstrating eosinophilic material in the sinusoid (arrow), portal area with perivascular inflammation (double arrow) and vacuolated hepatocytes (arrow head), (H&E, X:100). **Fig. (29):** Liver section from the 3rd group after two weeks of oral tramadol/APAP combination treatment displaying lose of architecture, interlobular hemorrhagic areas (arrow) and atrophied hepatocytes with pyknotic nuclei (arrow head), (H&E, X: 200). **Fig. (30):** Photomicrograph of liver tissue of 4th group after two weeks of oral APAP treatment showing expanded portal area, dilated congested portal vein (PV), perivascular inflammation (arrow), fibrosis (double arrow) (H&E, X:40). **Fig. (31):** Liver section from 4th group for two week of oral APAP demonstrating focal areas of necrosis (arrow), (H&E, X: 200). **Fig. (32):** Photomicrograph of liver tissue of the 2nd group (tramadol treatment) after 3-days of last treatment, displaying vacuolated hepatocytes (arrow) and restricted portal area (thick arrow). (H&E, X: 200). **Fig. (33):** Liver section from the 3rd group (tramadol/APAP treatment) after withdrawal period, demonstrating necrotic areas (arrow), Kűpffer cells (arrow head) and lymphocytes in sinusoids (thin arrow), (H&E, X: 200). **Fig. (34):** Photomicrograph of liver tissue of the 4th group (APAP treatment) after withdrawal period showing, central vein (CV) dilated congested sinusoids (arrow) and Kűpffer cells (arrow head), (H&E,X:200).

treatment showed 2 bands ranging from 1238.4 to 894.63 bp. While, the 3rd group of tramadol/APAP combination treatment displayed 3 bands ranging from 1234.6 to 844.3 bp. Meanwhile, the 4th group of APAP treatment showed one band 1219.4 bp.

The dendrogramatic analysis of DNAs profile of rat hepatocytes

The dendrogramatic analysis profile DNAs of rat hepatocytes revealed that the data obtained from that analysis after 1stweek of treatment regimens displayed similarity between the 1st group and the 2nd group with 95.02%, between the 1st group and the 3rd group with 94.25% and between the 1st group and the 4th group with 88.15%. While, after 2nd week of treatment regimens showed similarity between the 1st group and the 2nd group with 75.08%, between the 1st group and the 3rd group with 74.10% and between the 1st group and the 4thgroup with 84.24%. Meanwhile, after 3 days of last treatment regimens revealed similarity between the 1st group and the 2nd group with 81.23%, between the 1st group and the 3rd group with 91.36% and between the 1st group and the 4th group with 95.91% Fig. (36).

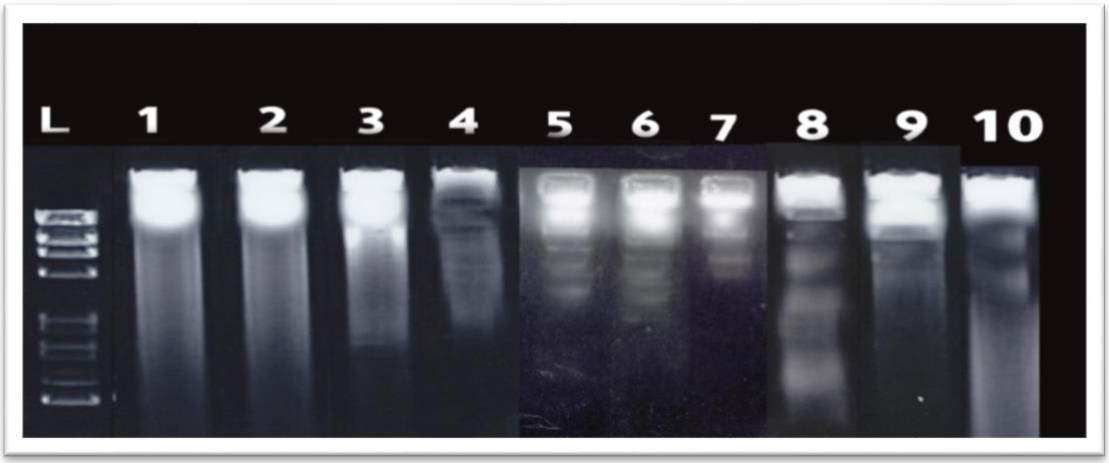

Fig. (35): Representive DNA-fragmentation profiles of rat hepatocytes treated with different doses of tramadol and /or APAP at different period of time.
L: Marker; Lane 1: 1st group (Control)
After 1st week of treatment: Lane 2: 2nd group; Lane 3: 3rd group; Lane 4: 4th group.
After 2nd week of treatment: Lane5: 2nd group; Lane 6: 3rd group; Lane 7: 4th group.
After withdrawal period: Lane 8: 2nd group; Lane 9: 3rd group; Lane 10: 4th group.

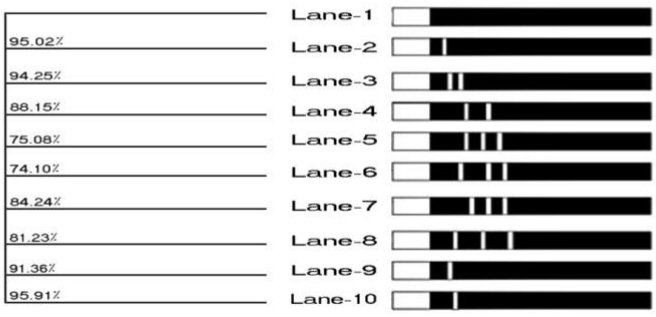

Fig. (36): Dendrogram derived from analysis DNA-fragmentation profiles of rat hepatocytes treated with tramadol and /or APAP after treatment and withdrawal period.

Table (1): DNA fragmentation pattern of rat hepatocytes treated with tramadol and /or acetaminophen at different period of time.

Lanes	Ladder		1st group		First week				Second week				3-days after last treatment			
					2nd group		3rd group		4th group		2nd group		3rd group		4th group	
band	bp	%	bp	%	bp	%	bp	%	bp	%	bp	%	bp	%	bp	%
1	1000	45.9	1804	100	1226.9	100	1190	100	1261.6	90.85	1729.6	76.1	1729.6	75.1	1729.6	75.3	1238.4	94.9	1234.6	95.4	1219.4	100
2	900	5.89							1044.3	4.78	1430.8	1.3	1401	14.4	1401	9.2	894.63	5.1	996.91	2		
3	800	5.58							844.3	4.33	979.15	3.49	773.73	10.5	800	15.38			844.3	2.6		
4	700	7.35									786.76	19.11										
5	600	5.46																				
6	500	2.62																				
7	400	9.22																				
8	300	3.68																				
9	200	7.5																				
10	100	6.8																				
Sum	100		100		100		100		100		100		100		100		100		100		100	
In lane	100		100		100		100		100		100		100		100		100		100		100	

Discussion

Repeated tramadol administration in patients might lead to the accumulation of toxic metabolites in the body, increase the risk for pharmacokinetic interactions, and/or decrease the clearance of tramadol, thus increasing its potential for toxicity. Previous study on 123 young multiple drug abusers found that seizures occurred in 26% of patients who combined marijuana and tramadol, and in 40% of those with hepatitis C (Grond and Sablotzki, 2004).

In present study, tramadol were administrated once a day for 15 days with increasing doses weekly following by 3 days of last treatment. The results of this study revealed that tramadol alone induced splenic reduction of white pulp with ill formed, splenic red pulp occupied large areas of splenic net work with predominant edema and megakaryocytes, suppressed phagocytic and killing of *S. aureus* by PMN and macrophage cells, decreased the production of Th1 cytokine (IFN-γ) in serum without any significant change inTh2 cytokine (IL-10), induced acute liver damage as result of tramadol toxicity, being vaculated hepatocytes with dilated sinusoid, proliferated Kupffer cells. Mild perivascular inflammatory response and inter lobular hemorrhagic areas were also detected. In addition to increasing doses, it increasd the inflammation reaction but its effective reduced after withdrawal period, exerted DNA changes in different regime of applications, which supported liver histopathlogical findings and explained by the dendrogramatic analysis profile DNAs of rat hepatocytes as following: 95.02%, 75.08% and 81.23% respectively. These results are consistent with intrathecal pumping higher dosage tramadol (50 microg/h) suppressed T lymphocyte proliferation and altered T lymphocyte subset phenotype but didn't affect NK cell activity, but, general analgesic dosage tramadol (25 and 12.5 microg/h) has no effect on the immune function (Zou *et al.,* 2008).

From previous studies histopathological examination of spleen after diacetylmorphine administration (1.625mg/100g b.wt.) showed depletion of lymphocytes in the white pulp follicle and decrease in its size, while, the cellularity of lymphocytes in red pulp increase (Afifi *et al.,* 1996). Moreover, splenocytes examination of treated male rats administration of tramadol (120 mg/kg) exhibited changes in the cellularity of lymphocytes in white and red pulps (Mostafa, 2006).

Moreover, several studies showed that opiates and opioids have been shown to interact with the immune system (Sacerdote *et al.,* 1997) and their suppressive effect on the phagocyte engulfing capacity has been well documented in a large number of publications (Kohnke *et al.,* 1999; Welters *et al.,* 2000). There are several studies attempting to elucidate the mechanism by which opiates alter the engulfing capacity of the phagocytic cells (Singhal *et al.,* 1998; Kohnke *et al.,* 1999). According to Makman *et al.* (1995) human granulocytes possess a morphine-sensitive μ3-receptor subtype that mediates a suppressive effect of the drug on the cell phagocytic capacity as well as modulation the generation of reactive species. It has been shown that naloxone is able to block this effect. Similarly, tramadol binds to μ-opioid receptors, but with low affinity (Sacerdote *et al.,* 2000). Another possibility is that morphine affects phagocytosis by inducing changes in cell membrane fatty acid composition causing a decrease of its fluidity, which in turn will impair formation of pseudopodia and inter-nalization of pathogens and foreign particles. A decrease in membrane fluidity of the blood-brain barrier tissue due to alteration in cell membrane fatty acid composition has been shown in rabbits following tramadol administration (Alici *et al.,* 2003).

In contrary of our results, other study showed that incubation of human peripheral blood phagocytic cells with increasing doses of tramadol affected neither the percentage of cells capable of phagocytosis, nor their phagocytic index (Beilin *et al.,* 2005). Another studies showed that ten days after the start of drug administration, the number of phagocytes and the phagocytic index reduced in morphine group and enhanced in tramadol

group (Liu *et al.,* 2006; Shirzad *et al.,* 2009).

Inflammation is a critical component of the overall pathophysiology, not only as a potential factor that may aggravate cell damage, but more importantly as a vital response to limit cell injury, remove cell debris and promote regeneration (Jaeschke, 2005).

IFN-γ is a Th-1-like, pro-inflammatory cytokine produced by activated T lymphocytes and natural killer cells. It boosts the production of IL-1 and IL-6. IL-10 is produced by Th2-like lymphocytes, B lymphocytes, and monocytes and it has important anti-inflammatory and immunosuppressive properties through suppression of IFN-γ and other proinflammatory cytokines. Therefore, the ratio of IFN-γ to IL-10 produced by immunocytes is of critical importance in determining their capacity to activate or inhibit monocytic and T-lymphocytic functions (Marta *et al.,* 2001).

Our data are similar to those of previous study showed that a remarkable Th2 differentiation after pretreatment with morphine or tramadol. IL-4 and IL-10 elevated significantly, while, IL-2 and IFN-γ decreased significantly compared with control groups. They also found that the suppression of cell-mediated immunity was dose dependent. It indicates that excessively high concentration of these drugs should be avoided in clinical applications in order to maintain a healthy Th1=Th2 balance. Th2 differentiation directed by morphine or tramadol was closely associated with the levels of corresponding cytokines, which was the cause of the immune alteration (Vallejo, 2004; Qian *et al.,* 2005).

The hepatotoxicity in albino rats of the present investigation may be attributed to dose-dependent. These histo-pathological finding coincide with those of El-Bourssali *et al.* (1993), Hemieda *et al.* (2007), El-wessemy, (2008) and Salem *et al.* (2010). In this regard, El-Bourssali *et al.* (1993) illustrated hepatotoxicity in albino rats after using tramal in case of daily treatment with single the therapeutic dose for a month, were including congestion and vascular dilatation of the hepatic blood vessels. The double dose revealed in addition to the preceding alterations, cellular infiltration and necrotic changes around the central vein. Further studies demonstrated that in case of daily treatment with double the therapeutic dose for a month, cloudy swelling, dilatation of hepatic sinusoids, scattered local areas of necrosis, thickened wall of the bile duct and increased number of hepatic binucleated cells was also observed. Furthermore, hepatic portal vein was severely damaged with the erosion of its epithelial lining cells was detected. The ultrasturctural alteration, include hypertrophy of the rough and smooth endoplasmic reticulum (RER; SER), such changes of RER affect protein synthesis indicated that in the aging process the oxygen metabolites react with unsaturated fatty acides in the phospholipids and cause damage to the plasma membrane and cell organelles such as the endoplasmic reticulum and lysosomes. There were also devastated mitochondria. This might be due to a defense mechanism exerted by the hepatic cells against the toxic effect of the drug keeping in consideration that the SER and RER have a detoxifying effect on toxins reaching any cell in the body (Hemieda *et al.,* 2007 and El-wessemy, 2008).

Another study demonstrated that the postoperative effects of morphine and tramadol on the histopathology of liver in rabbits which had undergone isoflurane anesthesia, hepatocyte degeneration, central vein dilatation, and mononuclear cell infiltration in the morphine and tramadol group were more severe than those of the control group. In addition, sinusoidal dilatation in tramadol group was more severe than that of the morphine group. These results suggest that morphine and tramadol may lead to some changes in liver tissue (Zuhtu Utku *et al.,* 2006).

Tramadol and APAP combination treated rats showed the same alteration in spleen and hepatotoxicity producing by tramadol alone decreased the cellularity of spleen but less effective than tramadol alone. The immunological parameter showed significant reduction in phagocytic

and killing of *S. aureus* by PMN and macrophage cells as well as the effective of tramadol alone. Moreover, the production of the Th1- related cytokine, (IFN-γ) was reduced, whereas, the concentration of the Th2- cytokine (IL-10) increased in serum after 1st week of treatment in dose dependent manner.

The results of this study revealed that tramadol/APAP exerted DNA changes in different regime of applications, which supported liver histopathlogical findings and explained by the dendrogramatic analysis profile DNAs of rat hepatocytes as following: 94.25%, 74.10% and 91.36% respectively. Subsequently, tramadol/ APAP treated rats appeared more effects on proinflammatory and anti-inflammatory mediator and fewer effects on DNA fragmentation of hepatocytes than tramadol alone.

The effects of APAP alone on treated rats were studied in dose dependent manner. Our results revealed that APAP alone induced the same alteration in spleen and hepatotoxicity of both tramadol alone and tramadol/APAP. Moreover, the immunological studies showed that its effects on cellularity of spleen were fewer than tramadol alone and tramadol/APAP but similar to its effects on the phagocytic and killing of *S. aureus* by PMN and macrophage cells. APAP decreased the production of Th1 cytokine (IFN-γ) in serum without any significant change in Th2 cytokine (IL-10), induced acute liver damage as result of APAP toxicity. APAP exerted DNA changes in different regime of applications, and explained by the dendrogramatic analysis profile DNAs of rat hepatocytes as following: 88.15%, 84.24% and 95.91% respectively. Subsequently to these results, it showed that tramadol/APAP induced the immunomedulatory effects more than tramadol and APAP alone.

The results in the present study are similar to several previous studies reported that cytological evaluation of splenic tissues after treatment with APAP showed thickened splenic septae, hyperplasia of monocytic - phagocyctic system, and erythrophagocytosis were appeared (Amy, 2005 and Salem *et al.,* 2010). Our results is coincide also with that observed by Shalabi**,** (1992) study, who demonstrated that the phagocytic activity of PMNs as tested for by the ingestion of opsonized dead yeast, was significantly reduced in APAP-treated cells. These results indicate clearly that APAP caused significant inhibition of the human PMNs function in vitro. From our histopathological findings, acute liver damage was observed as a result of APAP toxicity. These are in line with those of Salem *et al.* (2010) who reported that hepatotoxicity in dogs was detected as results of APAP toxicity (128 and 200mg/kg), severe congestation with disorganization of the hepatic cords, markedly vacuolated together with centrolobular necrosis, while, bile duct hyperplasia and congestion were predominant changes in the portal tract, centrolobular necrosis with massive degenerative changes in the hepatocytes was observed. Another results documented that the hepatotoxicity of APAP exposure may be attributed to increased the plasma levels of ALT, ALP, LDH, TNF-α and NO production. Moreover, APAP treatment reduced the glutathione level and antioxidant enzyme activities, increased lipid peroxidation and caused hepatic DNA fragmentation which ultimately leads to cellular necrosis. Also, incubation of hepatocytes with APAP reduced cell viability, enhanced ROS generation and increased CYP2E1 activity. APAP overdose caused injury in the hepatic tissue and hepatocytes via the upregulation of CYP2E1 and JNK (Hanawa *et al.,* 2008). These results indicated that APAP overdose caused hepatic injury due to its metabolism to hepatotoxic NAPQI (N-acetyl-p-benzoquinone imine), usually catalyzed by CYP2E1, and via the direct activation of JNK-dependent cell death pathway (Chun *et al.,* 2009; Das *et al.,* 2010; Han *et al.,* 2010).

The results of the present study consistent with these previous studies which demonstrated that in wild-type BALB/c mice, i.p. administration of acetaminophen (APAP 750mg/kg) induced intrahepatic IFN-γ mRNA expression and a marked increase in serum transaminase levels, leading to acute lethality of 45%. Histopathological examination showed centrilobular hepatic necrosis with

leukocyte infiltration and a large number of apoptotic hepatocytes 10 and 24 h after APAP challenge, mRNA expression of intercellular adhesion molecule 1, vascular cell adhesion molecule 1, interleukin (IL) 1 (IL-1), IL-6, tumor necrosis factor-α, monocyte chemoattractant protein 1, macrophage inflammatory protein (MIP) 1 MIP-2, KC, IP-10, Mig, Fas, and inducible nitric oxide synthase was enhanced in the liver of wild-type mice injected with APAP (Ishida *et al.,* 2002; James *et al.,* 2005). These observations suggested that IFN-γ may be involved directly in APAP-induced liver injury in addition to regulation of chemokine and adhesion molecule gene expression and subsequent inflammatory cell infiltration (Ishida *et al.,* 2002).

It is therefore revealed that acetaminophen-induced liver injury is associated with Th1-dominant response in Th1/Th2 cytokine balance (Masubuchi *et al.,* 2009). Considering the previous studies, a number of factors, including leukocyte infiltration, inflammatory cytokines, Fas-induced apoptosis, and NO, were probably responsible for the hepatotoxic mechanisms of APAP (Kamanka *et al.,* 2003; Hinson *et al.,* 2004). Alternatively, IFN-γ is a candidate target molecule in the therapeutic regimen of APAP-induced liver injury (Ishida *et al.,* 2004; Jaeschke, 2005).

Leukocyte infiltration, a hallmark of inflammation, is observed in most liver injuries caused by various types of chemical substances including APAP (Luster *et al.,* 2001). As neutrophil depletion or macrophage inactivation diminished APAP-induced liver injury, leukocyte infiltration is presumed to contribute to its development (Lawson *et al.,* 2000; Liu *et al.,* 2006).

The results of other study confirm that APAP-induced swollen sinusoidal endothelial cells (SECs) injury precedes hepatocellular injury, supporting the hypothesis that SECs are an early and direct target for APAP toxicity. These findings also suggested that reduced sinusoidal perfusion and increased Kupffer cell activity contribute to the development of APAP-induced liver injury (ITO *et al.,* 2003). Acetaminophen hepatotoxicity is the leading cause of drug induced liver failure. Several studies suggested that reactive metabolite formation, glutathione depletion, (Donnelly *et al.,* 1994; Abdelmegeed *et al.,* 2010), and alkylation of proteins, especially mitochondrial proteins, (Jollow *et al.,* 1973; Masubuchi *et al.,* 2005), were critical initiating events for the toxicity. Bcl-2 family members Bax and Bid (Chao and Korsmeyer 1998; Bajt *et al.,* 2005) form pores in the outer mitochondrial membrane and release intermembrane proteins (Jaeschke and Lemasters, 2003; Scorrano and Korsmeyer, 2003), e.g., apoptosis-inducing factor (AIF)(Susin *et al.,* 2000) and endonuclease G (van Loo *et al.,* 2001), which then translocate to the nucleus and initiate chromatin condensation and DNA fragmentation, respectively. Other effects of mitochondrial dysfunction, due to covalent binding, (Cohen *et al.,* 1997; Qiu *et al.,* 1998) leads to formation of reactive oxygen and peroxynitrite (Rogers *et al.,*1997) which trigger the membrane permeability transition and the collapse of the mitochondrial membrane potential (Burcham and Harman, 1991; Rogers *et al.,* 2000). In addition to the diminishing capacity to synthesize ATP (Jaeschke, 1990; Tirmenstein and Nelson 1990), endonuclease G and AIF are further released. Endonuclease G, together with an activated nuclear Ca2+, Mg2+ - dependent endonuclease (Tsokos-Kuhn *et al.,* 1988) cause DNA degradation (Nagata *et al.*. 2003), thereby preventing cell recovery and regeneration. Disruption of the Ca2+ homeostasis (Kim *et al.,* 2003) also leads to activation of intracellular proteases, e.g., calpains, which can proteolytically cleave structural proteins. Thus, multiple events including massive mitochondrial dysfunction (Jaeschke and Lemasters 2003; Scorrano and Korsmeyer 2003) and ATP depletion, extensive DNA fragmentation (El-Hassan *et al.,* 2003; Bajt *et al.,* 2005) and modification of intracellular proteins contribute to the development of oncotic necrotic cell death in the liver after acetaminophen overdose (Bajt *et al.,* 2005; James *et al.,* 2009).

Several studies demonstrated that APAP increased apoptosis of hepatocytes by up-regulating apoptosis signal-

regulating kinasen 1 and c-jun N-terminal kinase (Gunawan *et al.*, 2006;Nakagawa *et al.*, 2008), independently of peroxynitrite. APAP appeared to increase hepatic NO acting via mechanisms other than iNOS, this means that the metabolic activation as initiating event in the toxicity and mitochondrial dysfunction as the key cellular event that controls the propagation of the injury (Jaeschke and Bajt, 2006).

Therefore, based on the above discussion, it may be concluded that the effects the toxicity of the tramadol and /or APAP drugs in comparative study appeared several results. Firstly, the continuous use of these drugs during chronic treatment and withdrawal period in a dose dependence mannar by young addicts or patients may induce the immunomodulating effects. It translated by suppression of the phagocytic function of PMNc and macrophage cells in the peripheral blood, proinflammatory cytokine (IFN-γ) and increased anti-inflammatory mediator especially in tramadol/APAP treatment. In addition, few alterations appeared in splenocytes examination. It indicated that excessively high concentration of these drugs should be avoided in clinical applications in order to maintain a healthy Th1=Th2 balance. Secondly, these drugs may display severe pathological consequences of hepatocytes. These hepatic lesions may cause impairment of the liver function depending on induction of the cytochrome P-450 pathway, resulting in generation of ROS. In spite of its effective reduced during the withdrawal period. So, furthermore studies will be useful in understanding the details of the effective of tramadol/APAP on different parameter of immune system.

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دراسة مقارنة للتغيرات المناعية والهستوباثولوجية لعقار الترامادول ، ترامادول/ اسيتامينوفين و لأسيتامينوفين فى ذكور الفئران البيضاء

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قسم بحوث المخدرات - المركز القومى للبحوث الإجتماعية والجنائية- القاهرة

يعتبرعقارالترامادول من المواد الأفيونية الصناعية ويوصف كمسكن للآلام المتوسطة والشديدة ، وقد انتشرمؤخراً سوء إستخدام عقارالترامادول بين الشباب في معظم البلدان. وعرف الباراسيتامول (اسيتامينوفين) كعقارمضاد للالتهاب ومسكن ، وتؤدي زيادة جرعاته في كثير من الأحيان إلى الالتهاب الكبدى الحاد ، بينما يؤدي الترامادول و اسيتامينوفين معا (تراماست) إلى تأثير متكامل من المسكنات ، يتضمن بداية التأثير السريع للاسيتامينوفين والتأثير المستمر للترامادول . ولذا استهدفت هذه الدراسة إلى إلقاء الضوء على تأثير سمية العقاقير محل الدراسة على الجهاز المناعى فى ذكور الفئران البيضاء " دراسة مقارنة " لمدة إسبوعين ويلحقه ثلاث أيام من الا نسحاب . و قد تم تقسيم الفئران إلى أربع مجموعات ، استخدمت المجموعة الأولى كمجموعة ضابطة ، وأعطيت المجموعة الثانية جرعات بالفم عقارالترامادول والثالثة عقار الترامادول واسيتامينوفين معا والرابعة عقار اسيتامينوفين . بدأت الفئران المعالجة يوميا بجرعات عن طريق الفم على التوالى تبدأ من 45 ، 450/45 ،450 ملجم/ كجم من وزن الجسم في الأسبوع الأول وتنتهي 90 ، 900/90 ،900 ملجم/ كجم من وزن الجسم في الأسبوع الثاني ، وتم ذبح الفئران في نهاية الأسبوعين الأول والثاني من المعالجة واليوم الثالث من إنسحاب العقاقير محل الدراسة . أظهرت النتائج إنخفاض فى عدد الخلايا الليمفاوية فى الطحال للفئران المعالجة ، بالإضافة إلى ذلك أظهرت الدراسة النسيجية للطحال حدوث بعض التغييرات شملت اضطراب في تنظيم وتنسيق الخلايا الليمفاوية فى اللب الأبيض مع زيادة حجم اللب الأحمر، وزيادة عدد خلايا megakaryocytes فى اللب الأحمر للفئران المعالجة محل الدراسة. كما سجلت الدراسة انخفاض نشاط الخلايا البلعومية بالدم فى وجود الخلايا البكتيرية (*S. aureus*) مقارنة بالمجموعة الضابطة. وأظهرت نتائج الدراسة أيضا نقصا ملحوظا فى مستوى السيتوكين IFN-γ ، مع بقاء مستوى IL-10 دون تغير بالدم فيما عدا الفئران المعالجة بعقار الترامادول و اسيتامينوفين معا. أما الدراسة النسيجية لكبد الفئران المعالجة كشفت عن تغيرات مرضية تتضمن اضطراب تنظيم وتنسيق الخلايا الكبدية وتمدد الأوعية الدموية واحتقانها و كذلك ظهور فجوات سيتوبلازمية وتحطم فى الأنوية مثل التنكرز والتحلل، كما شوهدت مساحات من الإرتشاح الخلوى و عدد من الخلايا البلعمية (كوبفر). وقد دعمت هذه التغيرات بدراسة المحتوى النووى للخلايا الكبدية للفئران المعالجة بإستخدام طريقة التحلل الكهربى و التحليل الإحصائى (Dendrogramatic analysis) . وقد خلصت الدراسة إلى أنه يفضل استخدام عقار الترامادول/ اسيتامينوفين عن عقار الترامادول و اسيتامينوفين كل على حده مما له من تأثير على الجهاز المناعى فى زيادة مضادات الالتهاب ، ولكن تؤدي زيادة جرعاتهم الى اختلال كبدي لوظيفة الكبد . لذا يجب وضع ضوابط صارمة لاستخدام العقاقير محل الدراسة.