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Silymarin extract modulates toxicity, injury, oxidative stress and PCNA alternations induced by tramadol in rat liver

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Abstract

Tramadol is a synthetic opioid analgesic commonly prescribed for moderate to severe pain. This study was designed to evaluate the effects of silymarin supplementation on Tramadol induce injury, oxidative stress and PCNA expression alterations on liver in rats. For this purpose 40 male albino rats were divided into four groups and treated for 4 weeks (group 1 was control, group 2 was silymarin, group 3 was Tramadol and group 4 was Tramadol plus silymarin). The obtained results revealed that; serum GPT, GOT, ALP, GGT activities and MDA levels in liver tissues were significantly increase in rats treated with Tramadol as compared to control group while, total protein, albumin, globulin levels in serum, GSH, SOD and catalase levels in liver tissues levels were significantly decrease in Tramadol group when compared with control rats. Liver sections in rats treated with Tramadol exhibited mild positive reactions were detected for PCNA-ir, marked dilation or congestion in central veins, marked cellular infiltrations, atrophied and vacuolated hepatocytes. Treated rats with Tramadol plus silymarin succeeded to modulate these observed abnormalities resulting from Tramadol as indicated by the reduction of enzymes activity and the pronounced improvement of the investigated biochemical, antioxidant parameters, oxidative stress, hepatic injury and PCNA alterations. Further studies are needed to investigate the impacts of tramadol on human health.

Key Words: Opioid; Tramadol; Silymarin; Liver; toxicity; Oxidative stress; PCNA expression.

1 Introduction

Opioid is the term used broadly to describe all compounds that work at the opioid receptors (Shadnia et al., 2008). Tramadol is a synthetic opioid analgesic commonly prescribed for moderate to severe pain, (Niesters et al., 2013). Tramadol is structurally related to codeine and morphine, but it is 6000-times less potent than morphine and 10-times less potent than codeine (Matthiesen et al., 1998; Benini and Barbi, 2014). Tramadol usual doses being up to 200 mg/day while the maximum allowed daily dose is 400 mg. Tramadol is a synthetic opioid responsible for numerous adverse

effects including life-threatening ones such as loss of consciousness, dizziness, headache, somnolence, nausea, constipation, sweating, pruritus, seizures, serotonin syndrome and cardiovascular failure (Pothiawala and Ponampalam, 2011). Tramadol is well known under the trade names Tramacet, Ultracet, Tramapap, Amadol, Altram, Ultram and acetaminophen. Human deaths related to tramadol a demonstration have been reported, both when ingested alone in overdose and when taken in combination with potentially interacting drugs (Hawkes, 2013; Randall and Crane, 2014).

Herbal plants are used as medicines in folk and traditional medicinal practice based on the use of plants and plant extracts. People in all continents have used hundreds, if not thousands of indigenous plants for treatment of various ailments dating back to prehistory. Silymarin or milk thistle (*Silybum marianum*) is native to the Mediterranean, but now widespread throughout the world (Salama et al., 2015). Silymarin is a complex mixture of four flavonolignan isomers (silybin, silychristin, silydianin, and isosilybin) and acts as an antioxidant by reducing free radical production and lipid peroxidation, in addition to its antifibrotic activity (Abou zeinab 2013). Silymarin acts as a membrane stabilizer, has antioxidant properties, promotes regeneration of hepatocytes, decreases inflammation and prevents liver fibrosis (Feher and Lenqyel, 2012). Therefore; the present study was conducted to examine the possible modifying effects of silymarin aqueous extract against liver lipid peroxidation, enzymatic and non-enzymatic antioxidants, tissue injury, and PCNA alterations induced by Tramadol in male rats.

2. Materials and Methods

Tramadol tablets, each contains 225 mg tramadol hydrochloride (October Pharma Co., Giza, Egypt).

Silymarin seeds extract (*Silybum marianum*) was purchased from European Pharmacopoeia, Egypt.

2.1 Animal groups

The experiments were performed on 40 male albino rats weighing 180-200g and 12 weeks old. The rats were kept in the laboratory for one week before the experimental work and maintained on a standard rodent diet and water available *ad libitum*.

Rats were equally divided into four groups (n=10) and treated for 4 weeks. 1st control group in which healthy untreated rats; 2nd silymarin group in which rats receive silymarin orally (100 mg /kg BW/day); 3rd Tramadol group in which rats receive Tramadol orally (40 mg/Kg BW/day); 4th Co-treated Tramadol with silymarin include rats that receive orally tramadol and silymarin.

2.2 Sample Preparation

At the end of the experimental period (4 week), Animals were euthanized with intraperitoneal injection with sodium pentobarbital and subjected to a complete necropsy. Blood samples were individually collected from the inferior vena cava of each rat in non-heparinized glass tubes for estimation of liver functions (ALT, AST, total Protein, albumin, globulin, ALP and GGT).

2.3 Biochemical analysis

Both aspartate transaminase (AST) and alanine transaminase (ALT) activities according to Reitman and Frankel, (1957); Albumin and globulin concentration was estimated according to the method of Doumas et al. (1977); total protein concentration was estimated according to the method of Bowers and Wong (1980); alkaline phosphatase activity was estimated according to Belfield and Goldberge (1971) and GGT activity was estimated according to Szasz and Persijn (1974).

2.4 Enzymatic and Non-enzymatic Antioxidant Assays

Liver tissues were weighed, minced and homogenized (10% w/v) separately in ice-cold 1.15% KCl- 0.01mol/l sodium, potassium phosphate buffer (pH 7.4) in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 10,000 g for 20 min at 4°C, and the resultant supernatant was used for different enzyme assays. Malondialdehyde (MDA) was detected by TBARS analysis and measured as reported by Lahouel et al. (2004). Reduced glutathione (GSH) content was measured in liver homogenates after reaction with 5,5'- dithiobis-(2-nitrobenzoic acid) using the method of Ellman (1959). Superoxide dismutase enzyme activity (SOD; EC 1.15.1.1) in liver homogenate was assayed by the method of Misra and Fridovich (1972). Catalase activity in liver tissue supernatant was measured spectrophotometrically at 240 nm by calculating the rate of degradation of H₂O₂, the substrate of the enzyme. CAT activity was calculated as unit/mg protein (Aebi, 1984).

2.5 Histopathological and Immunohistochemical investigation

Immediately after decapitation rats were dissected, liver from different groups were quickly removed, washed in 0.9 saline solutions, fixed in 10 % neutral buffered formalin, dehydrated in an ascending series of alcohol, cleared in xylene and embedded in molten paraffin (mp. 50–58°C). Sections of 7 microns thickness were cut using rotary microtome and mounted on clean slides. Sections were stained with Ehrlich's haematoxylin and counterstained with eosin as a routine method after Bancroft and Stevens (1990).

Proliferating cell nuclear antigen immunoreactivity (PCNA-ir) was performed according to Tousson et al. (2011). Distribution of PCNA stained nuclei were examined in deparaffinized sections (5 µm) using an Avidin–Biotin–Peroxidase immunohistochemical method (Elite–ABC, Vector Laboratories, CA, USA) with mouse antibodies PCNA monoclonal

antibody (dilution 1:100; DAKO Japan Co, Tokyo, Japan).

2.6 Statistical Analysis: Data were expressed as mean values \pm SE and statistical analysis was performed using one way ANOVA to assess significant differences among treatment groups.

3. Results

No mortality was recorded during the experiment duration and all animals during the experiment time appeared healthy and did not show clinical signs of disease.

3.1 Serum markers of liver damage

Table (1) showed that; GPT, GOT, ALP and GGT activities were significantly increase in serum of rats treated with Tramadol as compared to control group ($P < 0.05$). Nevertheless all measured parameters showed a significant increase with silymarin co-treatment as compared with its Tramadol treated group (Table 1). On the other hand; total protein, albumin and globulin were significantly decrease in serum of rats treated with Tramadol as compared to control group ($P < 0.05$) while rats treated with Tramadol plus silymarin showed a significant increase in total protein, albumin and globulin concentration as compared to Tramadol treated groups.

Table 1: Changes in liver functions in different groups.

Parameters	Control	Sily	TR	Sily+TR
GOT (U/l)	133 \pm 5.19 ^b	124 \pm 4.41 ^b	148 \pm 4.55 ^a	135 \pm 5.08 ^b
GPT (U/l)	26.5 \pm 1.08 ^b	25.35 \pm 1.74 ^b	40.2 \pm 1.29 ^a	35.00 \pm 2.23 ^{ab}
ALP (U/l)	135 \pm 5.25 ^b	130 \pm 4.95 ^b	188 \pm 5.46 ^a	149 \pm 5.66 ^{ab}
Globulin (g/dl)	4.27 \pm 0.14 ^b	4.55 \pm 0.22 ^b	3.90 \pm 0.25 ^a	4.23 \pm 0.29 ^b
Albumin (g/dl)	3.68 \pm 0.29 ^b	3.82 \pm 0.19 ^b	2.95 \pm 0.19 ^a	3.21 \pm 0.20 ^{ab}
GGT (U/l)	26.4 \pm 1.26 ^b	24.05 \pm 0.99 ^b	29.8 \pm 2.45 ^a	26.6 \pm 1.50 ^{ab}
Total protein (g/dl)	7.44 \pm 0.55 ^b	7.51 \pm 0.76 ^b	6.85 \pm 0.57 ^a	7.05 \pm 0.48 ^{ab}

Data are expressed as mean \pm SE of 10 observations. Superscripts of different letters differ significantly ($p < 0.01$) from each other. ^bSignificantly different from Tramadol group. ^aSignificantly different from control (G_1) group. rats treated with silymarin (Sily), Tramadol (TR) and silymarin plus Tramadol (Sily+TR).

Table 2: Changes in MDA (nmole/g tissue), glutathione content (GSH; mmol/mg protein), superoxide dismutase (SOD; U/mg protein) and catalase (CAT; U/mg protein) levels in liver in different groups.

Parameters	Control	Sily	TR	Sily+ TR
MDA	28.0 \pm 1.07 ^b	24.9 \pm 0.83 ^b	47.4 \pm 1.56 ^a	36.2 \pm 1.05 ^{ab}
GSH	2.51 \pm 0.085 ^b	2.58 \pm 0.04 ^b	1.75 \pm 0.053 ^a	1.80 \pm 0.038 ^a
SOD	79.25 \pm 2.44 ^b	83.55 \pm 2.98 ^b	52.36 \pm 3.41 ^a	62.48 \pm 3.80 ^{ab}
CAT	51.08 \pm 2.25 ^b	55.45 \pm 1.66 ^b	29.34 \pm 1.29 ^a	49.65 \pm 2.97 ^b

Data are expressed as mean \pm SE of 10 observations. Superscripts of different letters differ significantly ($p < 0.01$) from each other. ^bSignificantly different from Tramadol group. ^aSignificantly different from control (G_1) group. Rats treated with silymarin (Sily), Tramadol (TR) and silymarin plus Tramadol (Sily+TR).

3.2 Oxidative stress markers

The data summarized in Table 2 indicates that, a significant ($P < 0.05$) increased in the liver MDA of Tramadol group (G_3) as compared with the control group. In the same time, data declared significant

decreased ($P < 0.05$) in the liver GSH, SOD and catalase levels of the Tramadol group as compared with the control group. On the other hand; a significant decrease in the MDA and significant increase in GSH, SOD and catalase levels in liver tissues in treated Tramadol plus silymarin when compared to the Tramadol group.

3.3 Histopathological changes

Histological examination of the liver of the control and silymarin groups exhibited the normal architecture of the hepatocytes with prominent round nuclei and eosinophilic cytoplasm, and few spaced hepatic sinusoids arranged in-between the hepatic cords (Figures 1A&1B). In contrast, liver sections of Tramadol group exhibited marked dilation or congestion in central veins, marked cellular infiltrations, atrophied and vacuolated hepatocytes (Figure 1C) Liver sections in treated with Tramadol plus silymarin group exhibited a good degree of improvement in hepatocytes where only mild vacuolated hepatocytes with mild cellular infiltrations were observed (Figure 2D).

3.4 PCNA expressions

The detection and distribution in PCNA immunoreactivity (PCNA-ir) in liver sections in the different groups under study were revealed in Figures (2A-2D). Liver section in control and silymarin groups shows strong positive reaction for PCNA-ir (grade 4) in hepatocyte nuclei (Figures 2A&2B), while mild positive reactions were detected for PCNA-ir (grade 1) in the liver sections in Tramadol rats group (Figure 2C). Moderate positive reactions for PCNA-ir (grade 3) were observed in liver sections of treated rats with Tramadol plus silymarin (Figure 2D).

4. DISCUSSION

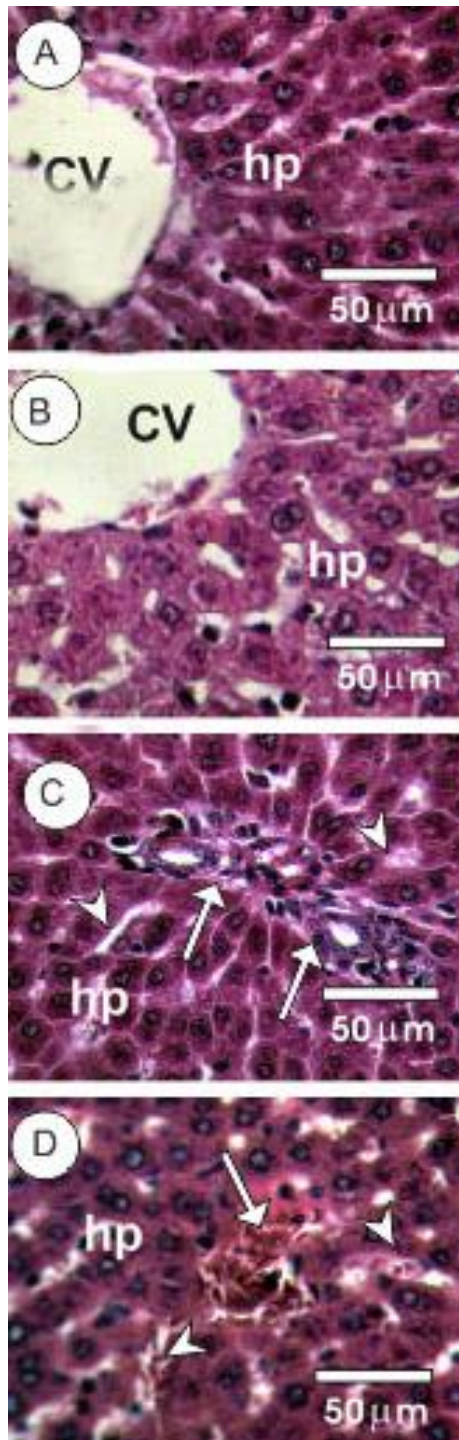
Tramadol belong to the same family of codeine, morphine and oxycodone (Neri et al., 2013; Niesters et al., 2013). Healthy liver is very important to overall health because liver plays a part in many important functions in the body including metabolism, bile secretion and detoxification by protection from exposure to foreign substances and eliminating them. For the determination of liver damage induced by Tramadol and the protective effect of silymarin, the activities of AST, ALT, ALP and GGT in serum in addition to protein, albumin and globulin were used as hepatotoxic biomarkers. Following treatment period of Tramadol administration, changes of enzyme activities indicating the occurrence of hepatic injury were observed. In the current study; GPT, GOT, ALP and GGT activities were significantly increase while the total protein, albumin and globulin were significantly

decrease after Tramadol administration. The reversal of increased serum enzymes in tramadol induced liver damage by the extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity.

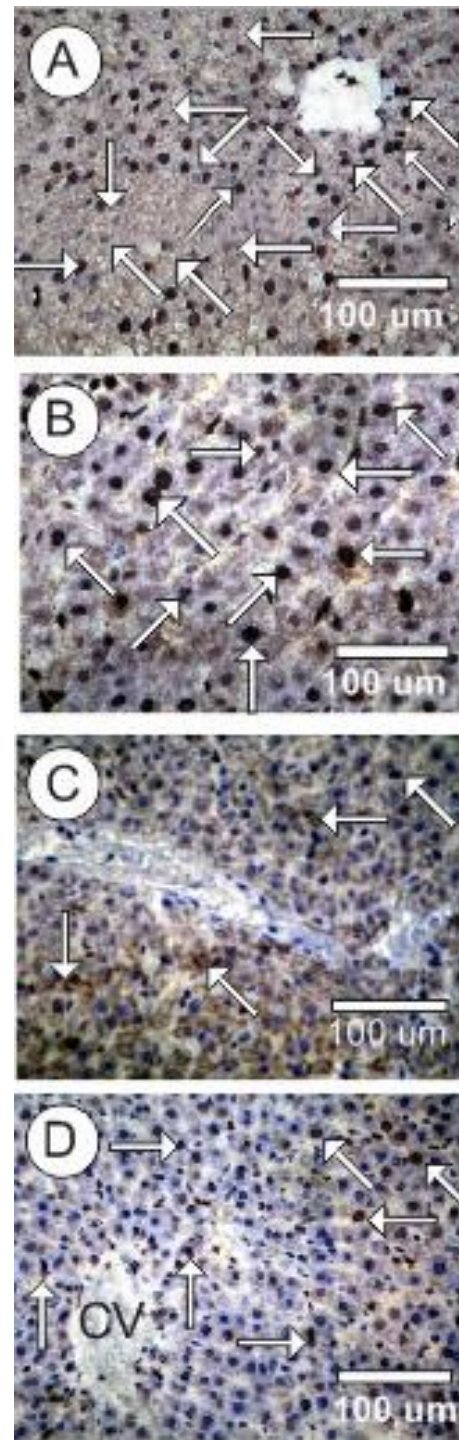
Our results agree with El-Gaafarawi (2006) who reported that a significant increase in the levels of GPT, GOT and LDH after tramadol administration. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Matthiesen et al., 1998). Shah et al. (2013) reported that; the liver is responsible for the metabolism and excretion of tramadol. Abdelmeguid et al. (2010) reported that; the elevations of liver enzymes are directly associated with higher concentrations of inflammatory markers and therefore it seems that the impact of silymarin on decreasing liver enzymes can be mediated by its suppressing effects on inflammatory biomarkers. Also; Drotman and Lawhan (1978) find the elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membranes in the liver. Borzelleca *et al* (1994) reported increased levels of ALT, AST and LDH in rats after long-term usage of morphine like agent levo-alpha-acetylmethadol (LAAM) HCL. Elyazji *et al.* (2013) reported that ALT, AST, LDH, Blood urea nitrogen (BUN) and creatinine were significantly higher in tramadol treated groups compared to the control group.

Because of its antioxidant properties, use of silymarin is beneficial in various chronic liver diseases caused by oxidative stress. Anti-inflammatory and diuretic properties of silymarin constituents enable the silymarin to protect the liver from dangerous toxins (Kiruthiga et al., 2007). In the current study; GPT, GOT, ALP and GGT activities were significantly decrease while the total protein, albumin and globulin were significantly increase in co-treated Tramadol with silymarin as compared with Tramadol treated group.

Oxidative stress, lipid peroxidation, and transaminase reactions are some of the mechanisms that can lead to liver dysfunction. Therefore, lipid peroxidation has been used as an indirect marker of oxidant-induced cell injury (El-Gaafarawi, 2006).



Figures 1: A-D: Photomicrographs of rat Liver sections in the different experimental groups stained with Haematoxylin & Eosin. **A&B:** Rat Liver sections in control and silymarin groups revealed normal structure of hepatocytes (hp). **C:** Liver sections of Tramadol group exhibited marked dilation or congestion in central veins (CV), marked cellular infiltrations (arrows), atrophied and vacuolated hepatocytes (White arrows). **D:** Liver sections in co-treated Tramadol with silymarin group exhibited a good degree of improvement in hepatocytes where only mild vacuolated hepatocytes (hp) with mild cellular infiltrations (White arrows) and mild cellular infiltration (arrows).



Figures 2: A-D: Photomicrographs of rat Liver sections in the different experimental groups stained with PCNA-ir. **A&B:** Strong positive reactions for PCNA-ir reaction in hepatocytes in control and silymarin respectively. **C:** Mild positive reactions for PCNA-ir in Tramadol group. **D:** Moderate positive reactions for PCNA-ir in Co-treated Tramadol with silymarin.

Toxic effects of opioids at the cellular level may be explained by lipid peroxidation. In the current study; a significant ($P<0.05$) increased in the MDA and a significant decreased ($P<0.05$) in the GSH, SOD and catalase levels in liver tissues in Tramadol group

when compared with control. Therefore decrease in the activity of catalase may result in a number of fatal effects due to the assimilation of superoxide radical and hydrogen peroxide. Masini *et al.* (1997) reported that; a significant increase in lipid peroxidation was reported in rats receiving an acute dose of cocaine. Similarly lipid peroxides were found significantly increased among chronic heroin users (Panchenko *et al.*, 1999). Our results agree with William *et al.* (1991) who find that; morphine exhibited a marked decrease in glutathione level when incubated with various concentrations of and resulted in cell death in the isolated rat hepatocytes. The increased levels of MDA and decreased levels of GSH, SOD and CAT suggest that excessive lipid peroxidation results in liver tissue damage and the failure of antioxidative defenses to mop up the excess production of ROS (Ranawat *et al.*, 2010). The administration of silymarin helped to ameliorate all these cellular changes by increasing the enzymatic antioxidants (SOD, CAT) as well as non-enzymatic antioxidant (GSH) and reducing the MDA level in the liver tissues. Our findings indicate that silymarin have the ability to scavenge the ROS to overcome the oxidative damage caused by Tramadol induced hepatic injury and that this recovery occurs after four weeks of co-treatment.

Medicinally, silymarin has been utilized for the treatment of liver diseases such as viral/drug induced hepatitis, cirrhosis and alcoholic liver disorders. Silymarin has also been shown an efficient role in the treatment of cancers. Mechanism of silymarin action includes prevention of binding of hepatotoxin to the specific receptor site on the membrane of hepatocytes, decrease in oxidation of glutathione to increase its concentration in liver and intestine, antioxidant property, stimulation of rRNA polymerase and increased protein formation, resulted in increased regeneration of hepatocytes (Nitin *et al.*, 2007).

The current study revealed that treatment with Tramadol induced marked dilation or congestion in central veins, marked cellular infiltrations, atrophied and vacuolated hepatocytes and decrease in PCNA immunoreactivity. In this regard, El-Bourssali *et al.* (1993) illustrated hepatotoxicity in rats after using Tramadol in case of daily treatment with single the therapeutic dose for a month, were including congestion and vascular dilatation of the hepatic blood vessels. Similarly, Nagmatsu *et al.* (1986) demonstrated that addition of morphine to the isolated rat hepatocytes induced a marked decrease in the cells and resulted in cell death. Our results agree with Rukhshanda *et al.* (2014) and El-Wessemy (2008) who demonstrated that; Tramadol

administration for long-term induced necrosis, vacuolization, central vein dilation, hemorrhage, cytolysis and complete cell membrane degeneration in mice hepatocytes. The liver histopathological effects of Tramadol toxicity in the current study were supported by the changes in the oxidative stress and liver function indices results. There were highly significant increase in serum AST, ALT, ALP enzymes and GGT levels after Tramadol administration when compared with control group. Silymarin with such effects and with its antioxidant property plays an important role in the treatment of iatrogenic and toxic liver disorders. Treatment with Tramadol plus silymarin succeeded to modulate these observed abnormalities resulting from Tramadol as indicated by the reduction of enzymes activity and the pronounced improvement of the investigated biochemical, antioxidant parameters, oxidative stress, hepatic injury and PCNA alterations. Our recommendation and conclusions, Tramadol have side effects and silymarin can ameliorate the biochemical changes, oxidative stress, and hepatic injury and PCNA alterations induced by Tramadol administration.

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