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Serum Paraoxonase and Arylesterase Activity and Liver Tissue of Rats under Acute Hepatotoxicity Induced By Acetaminophen

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ABSTRACT

If a significant relationship is proven between toxic dose of acetaminophen on liver tissue and paraoxonase and arylesterase activity of paraoxonase (PON1) and profiles of lipids and serum lipoproteins, this enzyme can be used as a diagnostic biomarker in acute liver injuries, including damage caused by toxic drugs such as acetaminophen. At the beginning of the experiment, the rats were randomly assigned to 6 groups taking acetaminophen (0, 500, 1000, 2000, 3000 and 4000 mg/kg); then, activities of paraoxonase, arylesterase, serum malondialdehyde, liver tissue, ALT level, liver index and HDL/LDL were measured. Paraoxonase and arylesterase activity was lower in serum of groups receiving acetaminophen than the control group. Similarly, activity of HDL/ LDL was lower in serum of groups receiving acetaminophen than the control group; however, MDA activity increased in serum of groups receiving acetaminophen compared to the control group. Moreover, activities of ALT and liver index increased in groups receiving acetaminophen compared to controls. The results showed that tissue MDA was higher in groups receiving acetaminophen than the control group and tissue paraoxonase and tissue arylesterase do not follow any particular trend. In this study, no significant relationship was found in toxic dose of

acetaminophen between intervention groups and controls and liver tissue, paraoxonase and arylesterase activity of paraoxonase (PON1) and HDL/LDL. Thus, this enzyme cannot be used as a diagnostic biomarker in acute liver injuries.

Keywords: Acetaminophen toxicity, Activity of paroxonase 1, Liver, Rat.

INTRODUCTION

Following skin, liver is the second largest organ and weighs 1200-1500 g in adults, accounting for one fiftieth of total body weight. Liver processes nutrients absorbed from gastrointestinal tract and stores for use by other organs. Therefore, liver acts as an intermediary between gastrointestinal tract and blood. Paraoxonase is an aryldialkylphosphatase [1], recently found in plasma of mammals. Paraoxonase first became famous in toxicology because of its ability in hydrolysis of xenobiotics such as paraoxon [2]. Paraoxonase has three families (isoforms) including PON1 (paraoxonase 1) and PON2 (paraoxonase 2) and PON3 (paraoxonase 3) [3]. PON1 and PON3 are generally found in human circulation, particularly with HDL (high-density lipoprotein) [2,3]. PON1 is an intracellular enzyme [4] and its physiologic function is not certain [5]. PON1 (paraoxonase 1) has esterase and lactonase activities and protects against xenobiotics (any foreign substance which enters the body and it is not produced naturally in the body, leaving beneficial or toxic effects) [6], while PON2 and PON3 have only lactonase activity [7]. All PONs are able to reduce oxidized LDL [8], while PON2 reduces cellular oxidative stress and prevents apoptosis in vascular endothelial cells [9]. Among PONs, PON1 is the best and most well-known [10]. PON1 is produced in liver, integrates with HDL in blood and improves antioxidant efficiency of HDL [11]. For this reason, measurement of enzyme activity is accompanied with measurement of lipid profile. Different studies have confirmed the role and significance of PON1 in pathogenesis of various diseases such as diabetes, chronic renal failure, obesity, metabolic syndrome, cancer, cardiovascular disease, Alzheimer, HIV infection and chronic liver disease [12,13]. Acetaminophen is widely used to relieve pain and reduce fever. However, acetaminophen overdose is known as one of reasons for liver damages and it is used to induce hepatotoxicity in scientific studies [14-16]. The present study examines serum paraoxonase and arylesterase activity and liver tissue of rats induced by acute liver toxicity caused by acetaminophen to determine changes in PON1 activity in serum and liver tissue after acute liver damage, show degree of liver damage in terms of PON1 activity and compare efficacy and changes in PON1 activity with other standard indicators of liver function test.

LITERATURE REVIEW

Studying 50 patients with acute liver disorder in India, Bindu et al. found that PON1 is known as a liver function test because of xenobiotic detoxification, evaluation of liver ability in excreting exogenous compounds, ability to synthesis and secretion [17]. By administrating CCl4 for 12 weeks for liver damage in rats, Marsillach et al. found that serum PON1 activity decreased, while serum PON1 concentration and hepatic protein expression increased; they suggested that PON1 might have a role in regulating apoptosis of liver cells [18].

Suleyman Sirri Kilic et al. studied 34 patients with chronic liver disease in Turkey. They found that reduced PON1 and arylesterase activity might: 1) help increasing liver disorder in chronic patients by reducing ability of HDL to LDL; 2) be a useful finding to assess hepatic failure in patients with chronic hepatitis caused by activity of this enzyme [19]. Palestinian Lipid Research Center reported that drug modulation of PON1 activity or PON1 gene expression could be a useful approach for prevention of atherosclerosis; moreover, enzyme secretion, enzyme turnover, protein stability, and level of PON1 gene

expression were a major determinant of PON1 status. This report summarizes recent advances in understanding PON1 expression in liver cells [20]. Studying acute and chronic hepatic and renal toxicity of NSAID (non-steroidal anti-inflammatory drugs), Eldin et al. found that oxidative stress-induced hepatic and renal functions were largely by acetaminophen more than Lornoxicom and Nimesulide, respectively, in both studies [21].

MATERIALS AND METHODS

Methods

Grouping rats

Wistar male rats weighing 170-230 g were brought from Laboratory Animal Resource Center of Ahvaz University of Medical Sciences and kept in animal rooms of Department of Pharmacy at 25 °C, 12 hours light and 12 hours of dark. At the beginning of the experiment, the rats were randomly assigned to 6 groups (n=5) taking acetaminophen (0, 500, 1000, 2000, 3000 and 4000 mg/kg).

Table 1 summarizes materials used in the experiments.

Producing acetaminophen solutions

Initially, carboxymethyl cellulose powder, Merck Co, existing in the laboratory was used to produce a 1% solution. Then, this solution was used to produce 400 mg acetaminophen. Serially, other concentrations of acetaminophen (0, 500, 1000, 2000, 3000 mg) were produced by the 4000 mg acetaminophen. At the beginning of the experiment, the rats were grouped, numbered and weighed. At the end of the experiment, the rats were weighed again. Their initial and final weights were compared. Based on weight of animals and test group, the required dose of acetaminophen was administered orally; after 2 hours of acetaminophen absorption, the animals were fed ad re-fasted. Blood samples were taken 2 hours later.

Table 1: The materials used in this study.

	Used material	Manufacturer	Country
1	Rat	Laboratory Animal Resource Center of Jondishapoor University	Iran
2	Acetaminophen	Elixir Pharmaceutical Factory	Iran
3	Tris buffer	Helena	Italy
4	Paraoxon	Sigma	USA
5	Phenyl acetate	Sigma	USA
6	Sulfuric acid	Kimiya	Iran
7	Thiobarbituric acid	Sigma	USA
8	Tetra ethoxy propane	Sigma	USA

Taking blood samples

The animals were anesthetized with ether; their chest was opened and about 2 cc blood was taken straight from their heart. After centrifugation, animal serum was kept at -70°C to measure hepatic enzymes and other biomarkers.

Taking liver samples

After taking blood samples, liver lobes were put on the chest slowly; using a forceps, junction of diaphragm to anterior lobe was pushed upward. The remaining liver connections were cut slowly by a surgical blade. The removed liver was weighed. Then, tissue samples were kept in bottles containing 10% formalin for 48 hours. A piece of liver tissue was removed for other measurements.

Measuring serum paraoxonase activity

A series of appropriate buffers and solutions including 1 mM Tris buffer, 1 mM CaCl2, measurement buffer, diluting buffer were produced for diluting paraoxon.

Experiments

Paraoxonase test

Initially, 20 µl diluted serum or plasma was transferred to a microplate to which 200 µl diluted paraoxon was added. It was shaken for 5 s and put in microplate reader device immediately. Absorbance (OD) was read every 15 s for 4 min at 405 nm wavelength. The refractive index was 18 $M^{-1}cm^{-1}$ for this reaction.

To measure serum arylesterase activity, 2 ml measurement buffer was poured in a quartz cuvette to which 10-µl serum was added. Contents of the cuvette were mixed several times and placed immediately in a spectrophotometer. Absorbance (OD) was read every 3 s for 33 s at 270 nm wavelength. Equation (1) was used to calculate serum arylesterase activity. The refractive index was 1310 $M^{-1}cm^{-1}$ for this reaction. Enzyme activity was calculated in mol/min/ml [22].

$$\text{Arylesterase activity}=\frac{change\ in\ absorbance\ at\ 270\frac{nm}{min}*1000*2(total\ volume\ in\ ml)*1000}{1310(molar\ extinction\ coefficicient)*1(cm\ patch\ length)sample\ volume\ in\ \mu\ l}$$

Measurement of Serum and Tissue Malondialdehyde (MDA)

Initially, 0.5 ml homogenate tissue existing in 10% Tris buffer solution or 0.5 ml serum was removed to which 1.5 ml 10% trichloroacetic acid was added and centrifuged for 10 min at 4000 g. Then, 2 ml 0.67% Thiobarbituric acid was added to 1.5 ml supernatant and incubated for 30 min in a boiling water bath. Five minutes after cooling the solution, the pink color caused by reaction of malondialdehyde with Thiobarbituric acid was evaluated by spectrophotometer at 532 nm wavelength. To draw calibration curve, different concentrations of tetraethoxy propane (1.5, 2.5, 5 and 10 mM) were used as standard. Results were expressed in mmol/g for tissue and nmol/ml for serum [23].

Measurement of tissue paraoxonase activity

Initially, 0.682 g Tris buffer was removed and weighed and poured in a 100 ml volumetric flask; then, 0.022 calcium chloride and distilled water was added to the flask (pH=8) to reach the volume 100. Next, 1 g liver was removed, sliced, homogenized by 9 cc Tris buffer for 2 min by using homogenizer device and centrifuged for 15 min at 1000 g. One ml supernatant was removed

and diluted with 10 ml Paraoxon (75 µl Tris buffer + 25 µl Paraoxon); it was incubated for 15 min at 37℃. The next steps are similar to measurement of serum paraoxonase activity [22].

Measurement of tissue arylesterase activity

Initially, 0.682 g Tris buffer was removed and weighed and poured in a 100 ml volumetric flask; then, 0.022 calcium chloride and distilled water was added to the flask (pH=8) to reach the volume 100. Next, 1 g liver was removed, sliced, homogenized by 9 cc Tris buffer for 2 min by using homogenizer device and centrifuged for 15 min at 1000 g. One ml supernatant was removed and diluted with 10 ml Paraoxon (85 µl Tris buffer + 15 µl Paraoxon); it was incubated for 15 min at 37℃. The next steps are similar to measurement of serum arylesterase activity [22].

Measurement of cholesterol, LDL and HDL

To determine Cholesterol, HDL and LDL levels, serum samples were delivered to medical diagnostic laboratory of Shafa Hospital and their values were measured using Pars Azmoon kit.

Measurement of ALT activity and histopathology

This enzyme catalyzes the following reaction:

Pyruvic acid + glutamic acid ⟷ alpha-Ketoglutaric acid + L-alanine

In conjunction with ALT enzyme activity, alanine glutamate is produced by adding saturating concentrations of substrate, which can be measured by glutamate dehydrogenase. This reaction transforms NADP to NAD+. Reduction of light intensity at 340 nm is proportional to ALT activity.

Liver tissue samples were delivered to laboratory in 10% formalin; the samples were stained, cut and fixated to produce plates. Photos were taken from plates under the microscope equipped with a digital camera. Liver histopathology results of the group receiving acetaminophen were compared to the control group.

Statistical tests

Since data was not normally distributed, the non-parametric Kruskal-Wallis test was used to compare results of different groups (P-value≤0.05). Additionally, DUNN'S test was used to compare differences between groups (Table 2).

Table 2: Table of variables.

Scale *	Practical definition of enzyme *	Qualitative		Quantitative		Dependent	Independent	Specifications
		Ordinal	Nominal	Discrete	Continuous			
µmol/lit/min	Paraoxonase Activity				*	*		Serum paraoxonase activity
mmol/lit/min	Arylesterase Activity				*	*		Serum arylesterase activity
µmol/g tissue/min	Paraoxonase Activity				*	*		Tissue paraoxonase activity
mmol/g tissue/min	Arylesterase Activity				*	*		Tissue arylesterase activity
mg/kg	Prescribed dose			*			*	Acetaminophen
nmol/ml					*	*		Serum MDA
nmol/g tissue					*	*		Tissue MDA

					*	*		ALT
mg/dl					*	*		Serum triglycerides
mg/dl					*	*		Cholesterol
mg/dl					*	*		LDL
mg/dl					*	*		HDL
Note: Practical definition of enzyme: amount of enzyme which can convert a micromoles of substrate to product per minute *Scale: enzyme unit in 1 ml (u/m)								

RESULTS

Liver index

At the beginning of the experiment, the rats were grouped, numbered and weighed. At the end of the experiment, the rats were weighed again. Their initial and final weights were compared. Kruskal-Wallis test showed that doses of acetaminophen had no significant effect on weight gain in rats. The results also showed that liver index increased in different doses compared to controls (Figure 1).

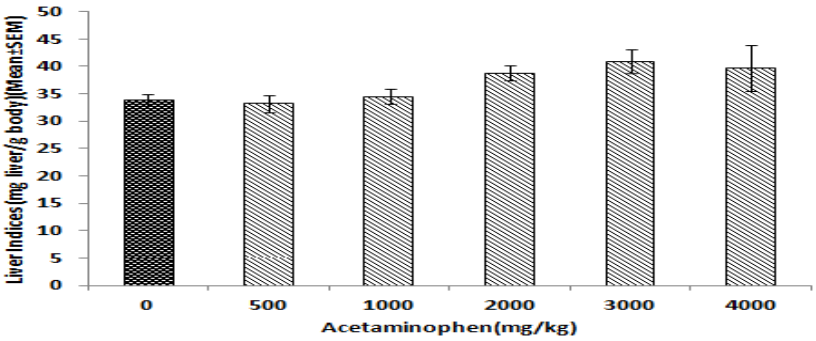

Figure 1: Liver index in mg liver/g body in male rats in different groups.

HDL/LDL

Since data was not normally distributed, Kruskal-Wallis test was used to compare HDL/LDL in different groups. Results showed that HDL/LDL decreased in different doses compared to controls; however, this reduction was not dependent on dose (Figure 2).

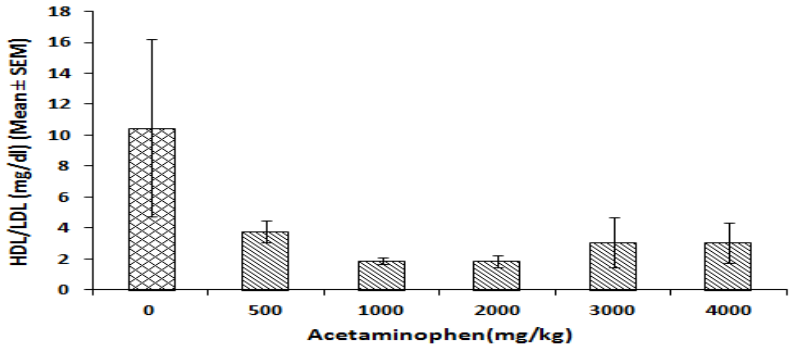

Figure 2: HDL/LDL (mg/dl) in male rats in different groups.

ALT activity

Since data was not normally distributed, Kruskal-Wallis test was used to compare ALT in different groups. The test showed a significant difference in ALT activity between different groups ($P<0.05$). DUNN's test showed a significant difference between controls and the group receiving 4000 mg/kg acetaminophen. The results also showed that ALT significantly increased in different doses compared to controls (Figure 3).

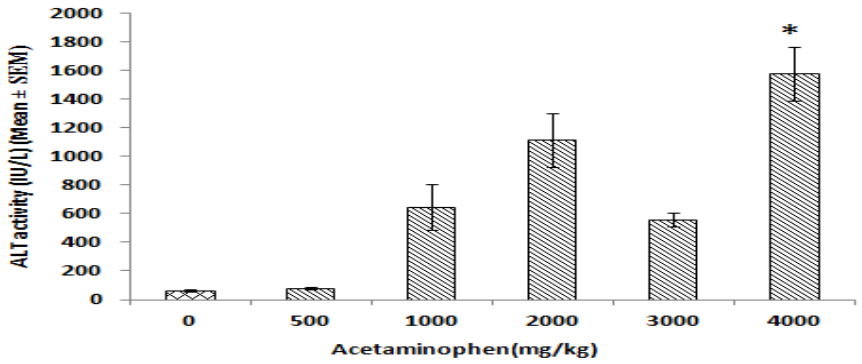

Figure 3: ALT (IU/L) in male rats in different groups (* significant difference between controls and group receiving 4000 mg/kg acetaminophen).

Serum PON1 activity

Kruskal-Wallis test was used to compare serum PON1 between different groups. The test showed no significant difference in serum PON1 between different groups. The results also showed that serum PON1 decreased in different doses compared to controls (Figure 4).

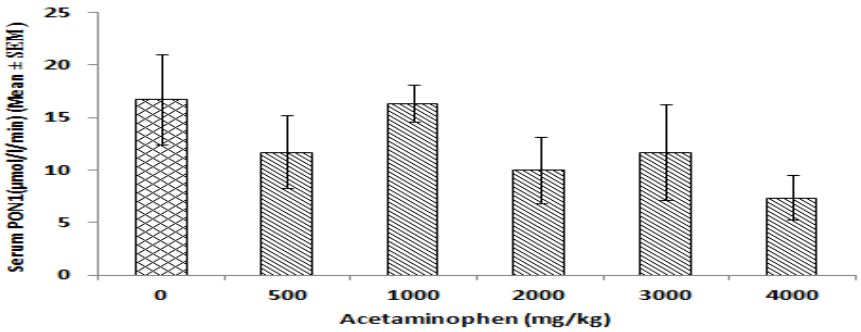

Figure 4: Serum PON1 (μmol/L/Min) in male rats in different groups.

Tissue PON1 sctivity

Kruskal-Wallis test was used to compare tissue PON1 between different groups. The test showed no significant difference in tissue PON1 between different groups. The results also showed that tissue PON1 did not follow a certain trend in different doses compared to controls (Figure 5).

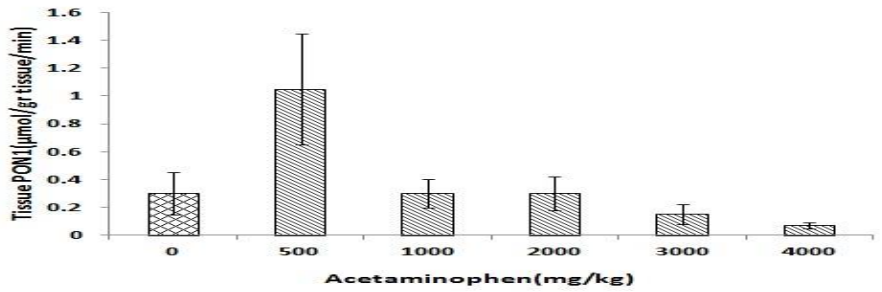

Figure 5: Tissue PON1 (μmol/G Tissue/Min) in male rats in different groups.

Serum arylesterase activity

Kruskal-Wallis test was used to compare serum arylesterase between different groups. The test showed no significant difference in serum arylesterase between different groups. The results also showed that serum arylesterase increased in different doses (except the group receiving 4000 mg acetaminophen) compared to controls (Figure 6).

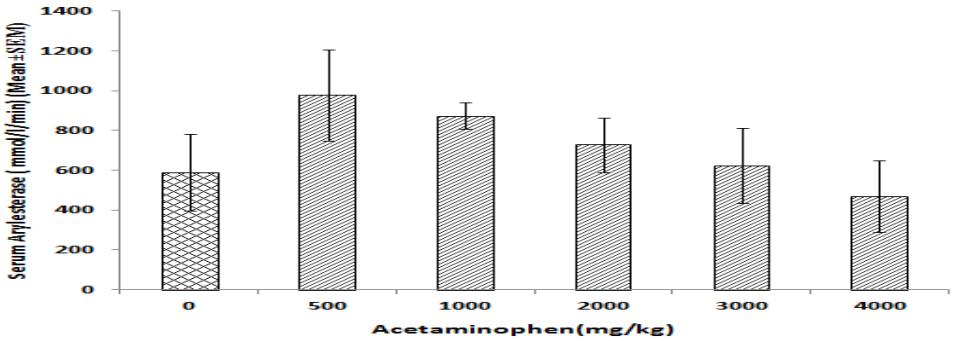

Figure 6: Serum arylesterase (mmol/L/min) in male rats in different groups.

Tissue arylesterase activity

Kruskal-Wallis test was used to compare tissue arylesterase between different groups. The test showed no significant difference in tissue arylesterase between different groups. The results also showed that tissue arylesterase did not follow a certain trend in different doses compared to controls (Figure 7).

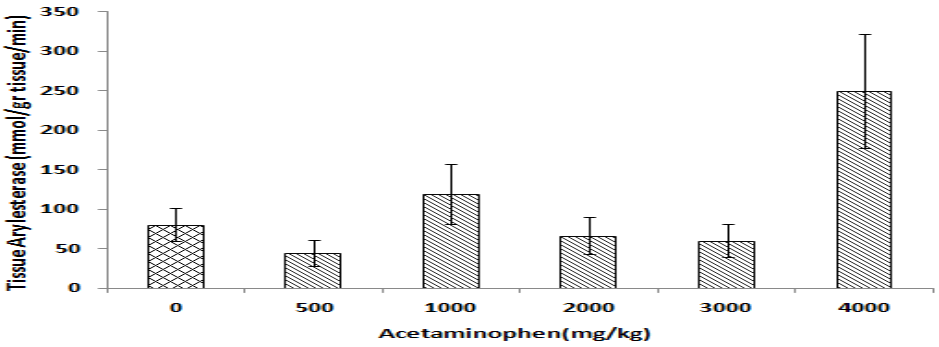

Figure 7: Tissue arylesterase (mmol/G tissue/min) in male rats in different groups.

Serum MDA

Kruskal-Wallis test was used to compare serum MDA between different groups. The test showed no significant difference in serum MDA between different groups. The results also showed that serum MDA increased in different doses compared to controls (Figure 8).

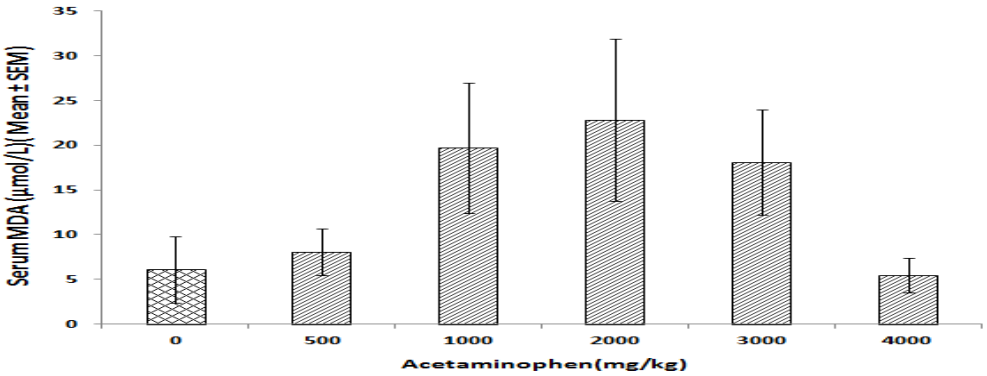

Figure 8: Serum MDA (μmol/L) in male rats in different groups.

Tissue MDA

Kruskal-Wallis test was used to compare tissue MDA between different groups. The test showed no significant difference in tissue MDA between different groups. The results also showed that tissue MDA increased in different doses compared to controls (Figure 9).

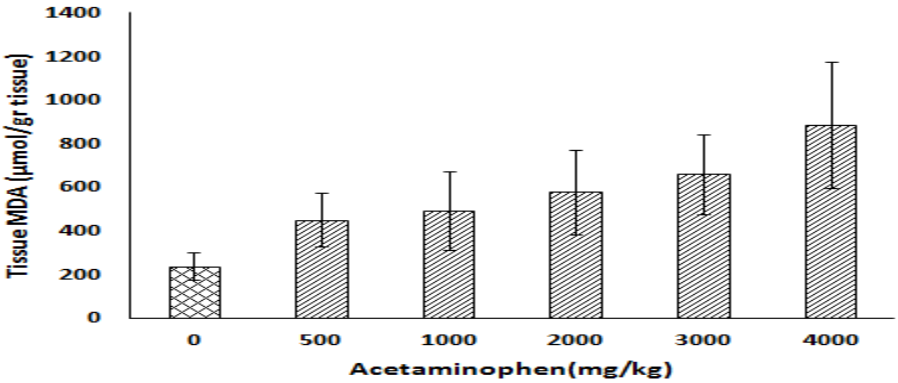

Figure 9: Tissue MDA (μmol/G Tissue) in male rats in different groups.

Relationship between liver index and HDL/LDL

There is an inverse relationship between liver index and HDL/LDL. The increase in liver index (body weight/liver weight) was associated with a decrease in HDL/LDL (Spearman correlation = 0.430; P-value<0.05) (Table 3).

Table 3: Relationship between liver index and HDL/LDL.

Correlations	index liver	HDL/LDL

Spearman's rho	index liver	Correlation Coefficient	1	-.430*
		Sig. (2-tailed)	.	0.022
		N	30	28
	HDL/LDL	Correlation Coefficient	-.430*	1
		Sig. (2-tailed)	0.022	.
		N	28	28
Note: *. Correlation is significant at the 0.05 level (2-tailed).				

Relationship between serum aryl-esterase and liver index

There is an inverse relationship between serum arylesterase and liver index. The increase in liver index (body weight/liver weight) was associated with a decrease in serum arylesterase (Spearman correlation = 0.450; P-value<0.05) (Table 4).

Table 4: Relationship between serum arylesterase and liver index.

Correlations			**serum aryl esterase**	**index liver**
Spearman's rho	serum aryl esterase	Correlation Coefficient	1	-.450*
		Sig. (2-tailed)	.	0.016
		N	28	28
	index liver	Correlation Coefficient	-.450*	1
		Sig. (2-tailed)	0.016	.
		N	28	30
Note: *. Correlation is significant at the 0.05 level (2-tailed).				

Relationship between serum arylesterase and HDL

There is a direct relationship between serum arylesterase and HDL. The increase in HDL was associated with an increase in serum arylesterase (Spearman correlation = 0.447; P-value<0.05) (Table 5).

Table 5: Relationship between serum arylesterase and HDL.

Correlations			serum aryl esterase	high dencity lipo protein
Spearman's rho	serum aryl esterase	Correlation Coefficient	1	.447*
		Sig. (2-tailed)	.	0.017
		N	28	28
	high dencity lipo protein	Correlation Coefficient	.447*	1
		Sig. (2-tailed)	0.017	.
		N	28	28
Note: *. Correlation is significant at the 0.05 level (2-tailed).				

Relationship between Tissue PON1 and Tissue MDA

There is an inverse relationship between tissue PON1 and MDA. The increase in tissue MDA was associated with a decrease in tissue PON1 (Spearman correlation = 0.820; P-value<0.05) (Table 6).

Table 6: Relationship between tissue PON1 and tissue MDA.

Correlations			Tissue Paraoxonase	Tissue Malondialdehyde
Spearman's Rho	Tissue Paraoxonase	Correlation Coefficient	1	-.820*
		Sig. (2-tailed)	.	0.046
		N	6	6
	Tissue Malondialdehyde	Correlation Coefficient	-.820*	1
		Sig. (2-tailed)	0.046	.

		N	6	6
*. Correlation is significant at the 0.05 level (2-tailed).				

DISCUSSION

Acetaminophen is usually used as an analgesic and antipyretic drug. High doses of acetaminophen can be fatal and toxic. In addition, accidental or intentional absorption of high doses cause hepatocellular necrosis which is associated with mortality [24]. In therapeutic doses, a part of acetaminophen is detoxified through glucuronidation and sulfation by liver and blood and excreted in the urine [15]. Another part of acetaminophen is oxidized by Cytp450 system to NAPQI; in normal conditions, this metabolite is detoxified and excreted through conjugation with GSH. Acetaminophen toxicity is one of the diseases in which ROS is believed to be involved. Lipid peroxidation caused by free radicals and mitochondrial damage caused by increased permeability causes liver toxicity induced by acetaminophen [25]. However, a study on rats showed that acetaminophen toxicity might vary depending on age and type of exposure [26]. In this study, mean tissue MDA increased in different groups receiving acetaminophen compared to control group. According to previous studies, this increase can be attributed to following reasons:

1. Acetaminophen led to NAPQI toxic metabolite in the body and thus lipid peroxidation and ultimately an increase in tissue MDA [26].
2. High doses of acetaminophen led to GSH depletion, decreased glutathione peroxidase activity and ultimately increased tissue MDA [27].

Serum MDA did not increase in 500 mg/kg dose compared to controls, while MDA increased in 1000 mg/kg dose possibly due to toxic metabolite of acetaminophen for compensation. Thus, higher doses of acetaminophen were associated with a reduction in MDA due to depletion of glutathione; this well indicates the curve shape [28]. In this study, HDL/LDL decreased in groups receiving acetaminophen compared to controls. According to the studies conducted, this can be attributed to liver damage due to acetaminophen exposure and reduced HDL production as well as impaired HDL secretion due to structural changes or changes in its value [29] and reduced PON1 level due to liver problems (necrosis) and increased LDL oxidation [20]. In this study, liver tissues of animals receiving different doses and control group were isolated and examined by pathology; the results showed that morphological cell changes were well visible even at low doses of acetaminophen; moreover, tissue necrosis and cell lysis were evident in 2000 mg/kg and 3000 mg/kg doses and 4000 mg/kg dose, respectively. This represents a very severe damage to liver tissue. However, previous studies showed that tissue necrosis did not develop in doses lower than 5000 mg/kg; this dose only disrupted cell arrangement [30]. One of the parameters used as a chemical biomarker to assess liver damage is ALT which is released by cellular damage from cytoplasm into blood and increases. ALT increase reflects hepatocellular damage. In this study, acetaminophen increased ALT, suggesting that acetaminophen induced damages on the liver cells; this is consistent with previous studies [30,31]. Very few studies have been conducted on PON1 enzyme activity assay in patients with acetaminophen toxicity [32], while no study was conducted in Iran. In this study, serum paraoxonase and aryl esterase decreased in different doses compared to controls; this is consistent with previous studies [33].

This study is one of the few studies where PON1 and MDA levels were measured together. This study found no significant difference between serum PON1 activity and oxidative stress in acetaminophen toxicity; this is consistent with previous studies [32,33]. This study examined the relationship between serum PON1 activity and ALT activity; no significant relationship was found between serum PON1 activity and ALT. This is consistent with previous studies [32,33]. Reduction mechanism of serum PON1 activity is not well understood in acetaminophen toxicity and can be driven by several factors. First, antioxidant action of

PON1 is associated with enzyme inactivation; through this process, free sulfhydryl group of PON1 reacts with specific oxidized lipids, and finally PON1 is inactivated probably due to invasion of free radicals (ROS) [34]. Other mechanism associated with reduced PON1 activity can be due to suppressed synthesis of enzyme caused by genetic defects or may arise from down regulation of transcription in liver [35,36].

Acetaminophen toxicity is different in different species of rats. Administration of the same dose of acetaminophen within 24 hours to different species of rat (Fisher and Sprague) causes different levels of ALT in them. Time of administration varies after taking blood samples; in this work, the considered time was 24 hours.

CONCLUSION

This study found no significant relationship between groups receiving toxic dose of acetaminophen and controls in liver tissue, serum paraoxonase and arylesterase activity of PON1, and HDL/LDL. Therefore, this enzyme cannot be used as a diagnostic biomarker in acute liver injuries, including damages caused by hepatitis and toxic drugs such as acetaminophen. One limitation of this study is that numerous parameters are involved in oxidant-antioxidant balance. This study only examined two parameters (PON1 and MDA). Therefore, generalization of results to total oxidant-antioxidant balance, regardless of other factors, may be followed by significant errors. In this regard, it is recommended to study other antioxidant agents such as vitamin E and C and Beta-carotene and other antioxidant enzymes such as SOD, catalase and glutathione peroxidase together with PON1 for better understanding of antioxidant properties of this enzyme. The number of tissue samples was low in this study; future works should use more samples. It is also recommended to examine the effect of long-chain phospholipids which lead to higher stability of PON1 on this enzyme by including lipids in diet and using dietary antioxidants simultaneously or separately.

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