EVALUATION OF XANTHINE OXIDASE ACTIVITY AND LIPID PEROXIDATION STATUSIN BOTH HEPATOPROTECTIVE AND HEPATOCURATIVE EFFECT OF THYMOQUINONE AGAINST CCL_4 TOXICITY

KHITHER H.[1*], MOSBAH A.[1], MADOUI S.[1], MOKHNACHE K.[1] and SOBHI W.[1]

[1]*Laboratory of Applied Biochemistry, Faculty of Nature and Life Sciences, Ferhat Abbas Setif -1- University, Setif 19000, Algeria.*

Abstract

The aim of the present study was to evaluate the *in vivo*influence of orally thymoquinone(TQ) hepatocurative and hepatoprotective treatments, on xanthine oxidase activityand lipid peroxidation status throughmalondialdehyde (MDA) level estimation, in plasma and liver homogenate, in male rats.

Hepatotoxicity was induced in rats by intraperitoneal injection of CCl_4at dose of 03 ml/kg, emulsified inolive oil (V/V), before or after orally treatment with TQ for 07 days, using two doses (2.5 and 05 mg/kg). Then, xanthine oxidase activity and MDA level wereevaluated in both plasma and liver homogenate of TQ-treated and no treated rats, using spectrophotometry methods.

This study revealed that CCl_4-induced hepatotoxicity is accompanied by a significant increase of xanthine oxidase activity with MDA level($p \leq 0.001$), inplasma and liver homogenate. In addition, TQ treatment led to restore xanthine oxidase activity and decrease MDA level, in a dose dependent manner, in both plasma and tissues homogenates.

These results suggest that Thymoquinone has asignificant *in vivo* hepatocurative and hepatoprotective effects through xanthine oxidase and lipid peroxidation inhibition properties.

Keywords: Thymoquinone, hepatotoxicity, CCl_4, lipid peroxidation, MDA, xanthine oxidase.

Introduction

Xanthine oxidase(XO; EC 1.1.3.22) is one of the endogenous source of Reactive Oxygen Species (ROS), through oxidation of hypoxanthine to xanthine and xanthine to uric acid. it requires the use of molecular oxygen as an electron acceptor, producing superoxide anion and hydrogen peroxide [1,2]. XO also catalyzes the reduction of nitrate to nitrite and NO• [2]. It has become the subject of great interest because of its involvement in several human pathologies. It is one of the most enzymes involving in inflammation and oxidative stress diseases, several studies have been published to investigate the role of xanthine oxidase in oxidative stress diseases [3,4,5,6].

Hepatotoxicity induced by CCl_4 is widely used, this hepatotoxicity model is the result of cytochrome P450-dependent reductive dehalogenation [7], during which CCl_4 induces liver damage in the rat following its biotransformation by the cytochrome P450 system into frees radicals. These highly toxic radicals can react with cellular macromolecules;proteins, DNA and membrane lipids then induce oxidation of unsaturated fatty acids of phospholipids present in the cell membrane, resulting in lipid peroxidation in hepatocyte membranes [8, 9].During lipid peroxidation, MDA is one of the final products of the decomposition of polyunsaturated fatty acids under the effect of radicals released during oxidative stressandxanthine oxidase activity.

Auteur correspondant: h.khither@yahoo.fr / khither.hanane@univ-setif.dz

Thymoquinone (TQ) is the major compound of the volatile oil derived from Nigella sativa seeds. It has several pharmacological properties; such as anti-inflammatory, antioxidant [10].

The aim behind this study is to evaluate the *in vivo*influence of orally thymoquinone(TQ) hepatocurative and hepatoprotective treatments as provided in our previous study [11], on xanthine oxidase activity as ROS generator enzyme, and MDA level as final product of lipid peroxidation, in plasma and liver homogenate, in male rats.

2. Materials and Methods

2.1. Chemicals

Thymoquinone,carbon tetrachloride (CCl_4), xanthine substrate, trichloroacetic acid (TCA), thiobarbituric acid (TBA)and all others products were purchased from Sigma Aldrich.

2.2. Experimental Animals

Fifty-six male Wistar rats (200–250 g) were purchased from the Animal House of Pastor institute Alger, Algeria. The animals were acclimatized for one week and maintained under standard conditions of temperature (23 ± 2°C),humidity (60 ± 10%) and 12 hours light/dark cycle. The rats were fed with a standard diet and water.

2.3. Induction of hepatotoxicity by CCl_4

Hepatotoxicity induced by intraperitoneal injection of CCl_4 is the most widely used model for studying liver toxicity in rats. The induction of hepatotoxicity is carried out according to the protocol of Wang and his collaborators (2004)[12]. Male rats are divided into seven groups of seven rats as follows:

Group 01 (normal): The rats in this group are treated by gavage of NaCl (0.9%)daily for 7 days.

Group 02 (Control): The rats in this group are treated daily, for 7 days by gavage of NaCl which contains 0.1% tween 80. On the seventh day, 1.5 ml / kg of olive oil are injected into the animals.

Group 03 (CCl_4): the rats of this group are treated by gavage of 0.9% NaCl, for 7 days. On the seventh day, they are injected with 03 ml / kg of CCl_4 previously diluted in 50% (V / V) olive oil.

2.3.1. Prophylactic treatments

Group 04 (Pro 2.5) and Group 05 (Pro 5): The rats in these groups are treated with gavage of 2.5 and 0.5 mg / kg of thymoquinone, respectively, for 7 days. On the 7th day, they are injected with 03 ml / kg of CCl_4, 30 min after the last dose of thymoquinone.

2.3.2. Curative treatments

Group 06 (Cur 2.5) and Group 07 (Cur 5): 03 ml / kg of CCl_4 are injected into the rats of this group on the 1st day, 30 min before the administration of the first dose of thymoquinone administered by gavage at a dose 2.5 and 0.5 mg / kg / day, respectively, for 7 days.On the eighth day, all the rats of the different groups are sacrificed under anesthesia with diethyl ether. The liver is immediately recovered, cleaned with sterile 0.9% NaCl and cold.

2.4. Blood sample

Blood samples are taken under anesthesia with diethyl ether from the retro-orbital sinus of the eye. The blood recovered in heparinized tubes is immediately centrifuged at 4000 rpm for 10 minutes. The sera are recovered and stored at -4 ° C until used for biochemical assays.

2.5. Preparation of the homogenate

After weighing the sample, the liver homogenate is prepared by homogenization of 500 mg of the liver in 5 ml of KCl buffer (0.15 M) at 4 ° C. The homogenates are centrifuged at 3000 rpm for 10 min and supernatants are aliquoted and then used for biochemical assays.

2.6. Determination of xanthine oxidase

The determination of xanthine oxidase is carried out according to the protocol of Bergmeyer et al. (1974)[13], following the increase in the production of uric acid at 290 nm formed following oxidation of xanthine in the presence of a source. enzymatic (plasma or homogenate). Briefly, 333 µl of xanthine (0.15 mM) are added to 666 µl of potassium phosphate buffer (50 mM, pH 7.5) and 33 µl of serum or homogenate in a quartz vat. Uric acid production is monitored by measuring the change in absorbance for 1 min against a blank that contains all reagents except the sample which is replaced by distilled water. The enzymatic activity of xanthine oxidase is calculated according to the following equation:

U / ml enzyme = (d A290 nm / min sample-dA290 nm / min White) (1) / (12.2) (0.033)

1 = the total volume.

12.2 = Milli-molar extinction coefficient of uric acid at 290 nm.

0.033 = the volume of the sample.

2.7. Determination of malondialdehyde level in both plasma and liver homogenate

The malondialdehyde (MDA) assay is used to estimate plasma lipid peroxidation and the spectrophotometrically homogenate at 532 nm. In acid and hot medium (pH 2 to 3, 100 ° C), an MDA molecule is condensed with two molecules of TBA to form a complex colored in pink. For the determination of MDA, 125 µl of homogenate or plasma are added to a mixture of 125 µl of trichloroacetic acid (20%) and 250 µl of thiobarbituric acid (0.67% TBA). The mixture is heated at 100 ° C for 15 minutes. After cooling, 1 ml of n-butanol is added followed by centrifugation at 3000 rpm for 15 minutes [14]. Absorbance is measured at 532 nm. The amount of MDA is calculated using the molar extinction coefficient of 1.56 x 105 M^{-1} cm^{-1} and the values are expressed in nmol of MDA formed per milliliter of plasma or per gram of the tissue.

2.8. Statistical Analysis

The data obtained were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test for all parameters and expressed as mean ± SEM. The p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of thymoquinone on xanthine oxidase activity

Xanthine oxidase (XO) activity in CCl_4-induced hepatotoxicity is evaluated in liver homogenate and plasma.The obtained results presented in figure 01 and Table 01 show that hepatotoxicity is accompanied by a significant increase in XO activity ($p \leq 0.001$) compared to that of the control group.In the liver homogenate, the XO activity measured in CCl_4 group of rats was 0.198 ± 0.006 U/mg protein, while the control group was 0.082 ± 0.015 U/mg protein.Similarly, plasma XO activity in CCl_4-treated rats was 0.189± 0.016 U/ml compared to 0.091 ± 0.017 U/ml in the control group.

In addition, prophylactic and curative treatments with 2.5 and 05 mg/kg/day TQ in rats intoxicated with CCl_4 cause a dose-dependent decrease in XO activity. In liver homogenatelevel, the curative treatment

with 05 mg/kg/day allowed to maintain the XO activity at a value comparable to that recorded in the control group (0.094± 0.016 U/mg protein compared to 0.082 0.015 U/mg protein).Similarly, prophylactic and curative treatments have led to a dose-dependent decrease in XO activity in plasma, but it remains higher than in the control group. The best decrease in XO activity is recorded with the healing treatment using 05 mg/kg/day of the TQ which gave an activity of 0.136± 0.008 U/ml compared to 0.091 ± 0.017 U/ml of control group.

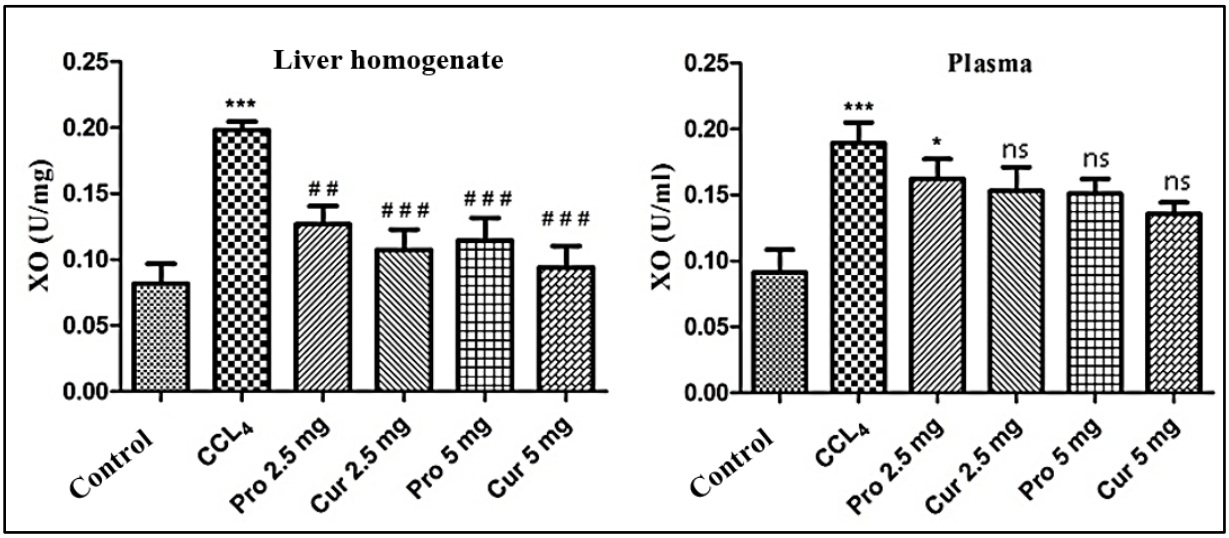

Figure 01: Effect of TQ on xanthine oxidase activity in CCl_4-induced hepatotoxicity. Values are expressed as a mean ± SEM, (n = 7); *: p≤0.05, ***: p≤0.001 a significant difference from the rat control group, # #: p≤0.01, # #: p≤0.001 a significant difference from the CCl_4 group.

Table 01: Effect of TQ on xanthine oxidase activity in hepatotoxicity induced by CCl_4.

Treatments	**Liver homogenat**	**Plasma**
	XO (U/mg de protéine)	**XO (U/ml)**
Control	0.082 ± 0.015	0.091 ± 0.017
Pro 2.5 mg	0.127 ± 0.014 $^{\#\#}$	0.162 ± 0.015 *
Pro 5 mg	0.114 ± 0.017 $^{\#\#\#}$	0.151 ± 0.011 ^{ns}
Cur 2.5 mg	0.107 ± 0.016 $^{\#\#\#}$	0.153 ± 0.018 ^{ns}
Cur 5 mg	0.094 ± 0.016 $^{\#\#\#}$	0.136 ± 0.008 ^{ns}
CCl_4	0.198 ± 0.006 ***	0.189 ± 0.016 ***

Values are expressed as a mean ± SEM, (n = 7); *: p≤0.05, ***: p≤ 0.001 a significant difference from the rat control group, $^{\#\#}$: p≤0.01, $^{\#\#}$: p≤0.001 a significant difference from the CCl_4 group.

3.2. Determination of malondialdehyde level in both plasma and liver homogenate

Hepatotoxicity induced by CCl4 is accompanied by a highly significant increase ($p \leq 0.001$) in MDA level; 174 ± 3 nmol/g tissue compared to 76.9 ±15.3 nmol/g tissue in the control group. Results of prophylactic pre-treatment and curative treatment of rats with TQ using 2.5 and 05 mg/kg/day for 7 days showed that TQ significantly decrease ($p \leq 0.001$) the rate of MDA levelcompared to the CCl_4 group. The most significant decrease is observed with the curative treatment either for the 2.5 dose or that of 05 mg/kg/ml.

Assay results for plasma MDA level showed a significant increase ($p \leq 0.001$) in MDA was observed in rats treated with CCl_4 (3.42 ± 0.43 nmol/ml compared to 0.97 ± 0.13 nmol/ml in the control group). In contrast, treatment and pretreatment with TQ in rats intoxicated with CCl_4 restored and maintained plasma levelof MDA ($p \leq 0.001$) at values very close to those observed in the control group. This restoration was dose-dependent (Figure 2 and Table 2).

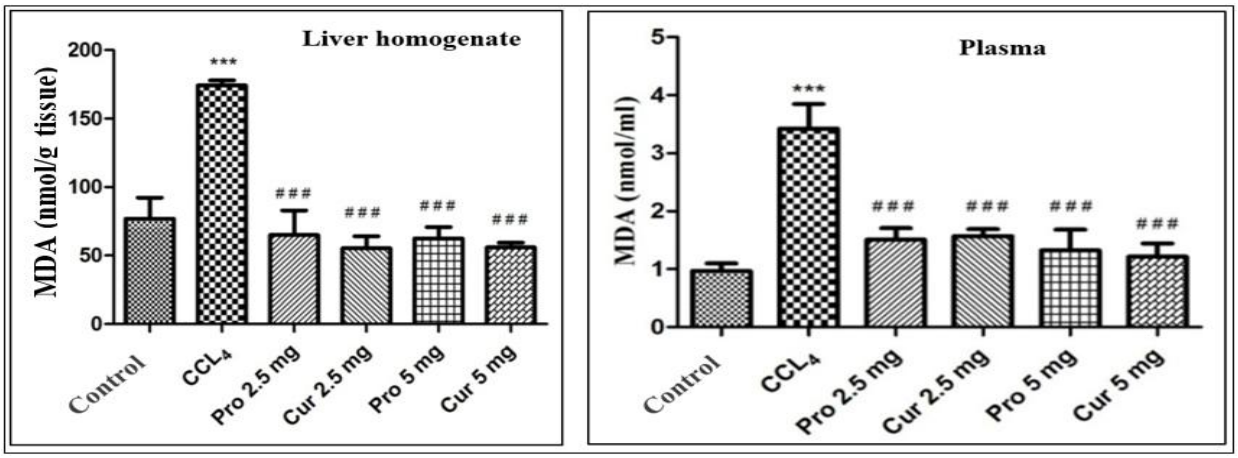

Figure 02: Effect of thymoquinone on liver and plasma MDA level.Values are expressed as an average SEM, (n = 7);**: $p \leq 0.01$, ***: $p \leq 0.001$ a significant difference from the rat control group,$^{###}$: $p \leq 0.001$ a significant difference from the CCl_4 group.

Table 02: Effect of thymoquinone on liver and plasma MDA level.

Treatments (mg/kg)	Plasma	Liver homogenat
	MDA (nmol/ml)	**MDA**(nmol/g Tissue)
Control	0.97 ± 0.13	76.9 ± 15.3
Pro 2.5 mg	1.51 ± 0.20 $^{###}$	64.9 ± 18.1$^{###}$
Pro 5 mg	1.32 ± 0.36 $^{###}$	62.3 ± 8.36$^{###}$
Cur 2.5 mg	1.57 ± 0.12 $^{###}$	55.2 ± 8.81$^{###}$
Cur 5 mg	1.22 ± 0.23 $^{###}$	55.9 ± 3.43 $^{###}$
CCl_4	3.42 ± 0.43 ***	174 ± 3.71***

The values are expressed as an average SEM, (n = 7);*: p≤ 0.05, **: p≤ 0.01, ***: p≤ 0.001 a significant difference compared to the control group of rats, $^{\#}$: p≤ 0.05, $^{\#\#}$: p≤ 0.01, $^{\#\#\#}$: p≤ 0.001 a significant difference compared to the group treated with CCl_4.

4. Discussion

Hepatotoxicity induced by CCl_4 is widely used as a model for the study of experimental liver damage in rats, since the liver is the main site of its biotransformation. This hepatotoxicity model is the result of cytochrome P450-dependent reductive dehalogenation [7], during which CCl_4 induces liver damage in the rat following its biotransformation by the cytochrome P450 system into trichloromethyl (CCl_3). It is a highly reactive free radical, which reacts rapidly with molecular oxygen to produce trichloromethyl eroxyl (CCl_3O_2). These highly toxic radicals can react with cellular macromolecules;proteins, DNA and membrane lipids then induce oxidation of unsaturated fatty acids of phospholipids present in the cell membrane, resulting in lipid peroxidation in hepatocyte membranes [8, 9], thus disrupting the homeostasis of Ca^{2+} that causes liver cell destruction [15].

The evaluation of xanthine oxidase activity in the case of hepatotoxicity is performed for the first time in this work.The obtained results in either homogenate or plasma indicate a significant increase in XO following the poisoning of rats by CCl_4. This can be explained by the transformation of the XOR to XO. Once XOR is released into the plasma, it is rapidly converted into a potential oxygen reactive XO oxidase form [16].

During oxidative stress disease and inflammation, xanthine oxidoreductase (XOR) is activated by different inflammatory mediators, such as TNF-α and IL-1, which cause xanthine deshydrogenase (XDH) to be converted to XO [1,3,17]. Activated neutrophils also cause irreversible conversion of the XDH form into the XO form in endothelial cells [18].

Curative and prophylactic treatment has significantly reduced XO activity. This effect could be explained by the antioxidant and anti-inflammatory effect of TQ. The latter can be rendered to its effect on cytokines involved in the activation of the conversion of XDH to XO. Indeed, Al-Malki and Sayed (2014)[19]have shown that TQ induces the reduction of TNF-α, IL-1 in a Cisplatin-induced hepatotoxicity model.

Lipid peroxidation in CCl_4-inducing hepatotoxicity is estimated in this study by the evaluation of MDA level.The results show that poisoning of rats with CCl_4 led to a highly significant increase in the level of MDA, which recorded in liver homogenate and plasma. The increase in MDA reflects a strong oxidative stress that leads to tissue damage and a failure of antioxidant defense mechanisms. MDA is considered as marker of lipid peroxidation. It causes loss of fluidity and membrane integrity and ultimately loss of hepatocyte function [20].

The results of this study show that the level of hepatic and plasmatic MDA decreased significantly following curative and prophylactic treatment with TQ. These results indicate that TQ can protect the liver against CCl_4 toxicity. This protection may be due to inhibition of lipid peroxidation [21]. The results are

consistent with those ofNagi et al. (1999)[22] and Nili-Ahmadabadi et al. (2011)[23], which showed a significant decrease in MDA following treatment with TQ against CCl_4-induced hepatotoxicity in rats.

Finally, the significant hepatocurative effect of TQ, without forgetting its important hepatoprotective effect, could be explained by its antioxidant effect, either by its scavenger effect of free radicals, inhibitor of lipid peroxidation and by its ability to induce a positive regulation of the antioxidant system (CAT, SOD and GSH) that failed due to oxidative stress during CCl_4-induced hepatotoxicity in rats.Hepatoprotective and hepatoregulatory effects may also be induced by the anti-inflammatory effect of TQ demonstrated in several studies. Negative regulation of xanthine oxidase activity may be involved in the hepatoprotective and hepatoocuratic effect of XO generating ROS.

Conclusion

This study, allows us tosuggest that Thymoquinone has the ability to improve significantly the xanthine oxidase activity and it is a significant lipid peroxidation inhibitor,in plasma and tissues. These results explain the significant *in vivo*hepatocurative and hepatoprotective effects through xanthine oxidase and lipid peroxidation inhibition properties of thymoquinone.

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