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Effects of Camphor on Hepatic Enzymes, Steroids and Antioxidant Capacity of Male Rats Intoxicated with Atrazine

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Abstract: This work aimed to determine if camphor oil has any protective properties to atrazine induced toxicity. To investigate this purpose, 40 Swiss albino male rats of 10 weeks old and 80-120g body weight were equally divided into four groups. Atrazine group orally administered 200 mg/kg, camphor group orally administered 30 mg/kg, atrazine and camphor group received 200mg atrazine and 30mg camphor on each other day for 30 days. Blood samples were collected from the retro orbital venous plexus of all animals before scarified and liver tissues were collected and preserved at -80°C. Plasma ALT, AST, SOD, MDA and total antioxidant capacity were measured in addition to testosterone and estradiol. Genomic DNA fragmentation was performed to hepatic tissues of all groups. Results revealed that treatment with camphor and/or atrazine or both increased levels of AST and decreased levels of ALT. Camphor oil decreased both testosterone and estradiol levels but atrazine increased testosterone levels. Camphor increased the aromatization of tesosterone to estradiol in atrazine and camphor group. Camphor alone decreased MDA and SOD levels but its combination with atrazine increased them also genomic DNA fragmentation was similar in both atrazine treated groups and camphor showed no protective effects.. In conclusion camphor has antioxidant properties but could not reduce the toxic effects of atrazine in rats.

Key words: Camphor · Atrazine · DNA Fragmentation · Antioxidants · Steroid Hormones · Rat

INTRODUCTION

The herbicide atrazine (2-chloro-4-(ethylamino)-6(isopropylamino)-s-triazine; ATR) is a very widely used broad-spectrum pesticide and is applied on major crops, such as corn, soybeans and sugarcane [1]. Besides its widespread use, an additional concern is the fact that ATR is the most frequently detected pesticide in ground and surface waters [2]. Many of the toxicological studies conducted so far have focused primarily on the effects of ATR on the endocrine and reproductive systems [3-5]. One prominent effect observed following ATR exposure is the disruption of the hypothalamic control of pituitary ovarian function, possibly by affecting hypothalamic dopamine (DA) and norepinephrine (NE) which are regulatory molecules for pituitary prolactin and luteinizing hormone secretion, respectively [3]. ATR decreases tissue DA levels not by affecting Tyrosine hydroxylase activity, but possibly by interfering with the vesicular storage and/or cellular uptake of DA [6]. Rats treated with ATR at a concentration of 400 mg/kg/day for 14 days increased catalase levels and maintenance of the expression of antioxidant enzymes (SOD). In addition, lipid peroxidation, hepatic tissue degeneration, activation of HSP90, increased levels of connexin mRNA and genotoxicity were observed. ATR induced early hepatic oxidative stress that triggered defense mechanisms to maintain the morph physiological integrity of the liver [7]. Exposure to atrazine and its primary metabolites such as diaminochlorotriazine has been reported to affect liver function in rats [8, 9]; testosterone secretion can affect reproductive function through suppression of gonadotropin-releasing hormone (GnRH) or LH release [10]. In addition, it produced necrobiotic changes and vacuolation of spermatogonial cells in testicular tissue of goat [11,12] inhibited the preovulatory surge of luteinizing hormone (LH) in rodent models [13], delayed puberty onset [14] and reduced testosterone levels in males [10].

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Camphor is a ketone white crystalline substance, obtained from the tree *Cinnamomum camphora L.,* commonly known as Camphor tree (Camphor wood or Camphor laurel), or produced synthetically. Its synthetic form is now available and is being produced for medical, sanitary and industrial usages [15-17]. Camphor has been used as aphrodisiac, contraceptive, abortificient, cold remedy, antiseptic and suppressor of lactation [18] Camphor may affect sexual activity [19]. Contrary, camphor at a dose 50mg/kg enhanced the sexual behavior and performance which might be due its effects in serum testosterone levels on modulations of sympathetic nervous system [20]. Camphor has antioxidant properties and its administration to male rats exposed to atrazine and its metabolites resulted in significant structural changes including vascularization and proliferation of sexual cells and affected maturation of seminiferous tubules and subsequently improving reproductive function of testes in mice [19]. Camphor play a role in improvement of immune function [21], enhancement of enzymatic breakdown of carcinogens [22] and the increased susceptibility of cancer cells to radiation [23]. Camphor ingestion may lead to abortion as it crosses the placenta and fetuses lack the enzymes to hydroxylate and conjugate with glucuronic acid [24]. Oral administration of different concentrations of camphor solution to rabbits for a period of ten days, resulted in mild edema, glomerulonephritis, glomerular lobulations, tubular necrosis and congestion of the blood cells [25].

Since ATR and its metabolites have been shown to affect reproduction of animals. Camphor treatment ameliorated the effects of atrazine suggesting it as a potential antioxidant against atrazine-induced oxidative stress. Thus, the main objective of this study is to evaluate the effects of camphor on liver enzymes, steroids and antioxidant capacity of male rats intoxicated with atrazine.

MATERIALS AND METHODS

Animals and Experimental Design: Forty Swiss albino male rats (10 weeks old, 80 to 120 gram body weight) kept under standard conditions of temperature, humidity and living regimens, with *ad libitum* access to food and water were maintained for one week accommodation period before conducting this experiment. Rats randomly divided into four equal groups (N=10 animals): the first group received no supplementation and served as a control group, the second group was supplemented orally with commercial atrazine at dose 200 mg/kg body weight, each other day for 30 days, the third group was supplemented orally with 30 mg/kg body weight of camphor oil, purchased from Oil Extraction and Press Unit, National Research Center, Egypt, for 30 days, while the fourth group was supplemented orally as groups two and three (200 mg/kg atrazine and 30 mg/kg camphor oil) for 30 days.

Blood and Tissue Samples: Blood samples were collected (In heparin containing tubes) from the retro orbital venous plexus of all animals before scarified and centrifuged at 3000 rpm, for 10 minutes. The clear supernatant plasma was harvested and kept at –20°C until hormonal and antioxidant assays. All rats were scarified at the end of the experiment, liver tissues were collected, washed in distilled water and stored at -80°C till be used for DNA extraction to assess the level of DNA damage.

Liver Enzymes Measurements: Plasma aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured [26] using commercial supplied kits (Biodiagnostics, Egypt).

Hormone Assaying: Estradiol (E2)and testosterone levels were assayed using commercial ELISA kit (DSI S.r.l., Italy). Sensitivity, intra- and inter-assay coefficient of variation was 10pg/ml, 9.1 and 9.8% for estradiol and 0.2nmol/L, 5.6 and 6.5% for testosterone, respectively.

Antioxidant Markers Measurements: Lipid peroxidation product (Malondialdehyde, MDA) was assayed using commercially supplied kits (Bio-diagnostic, Kit number MD2529). Total antioxidant capacity (TAC) was evaluated spectrophotometrically using commercially available kits. Superoxide dismutase SOD activity was measured using kits from Biodiagnostic, Egypt.

DNA Fragmentation Analysis: High quality genomic DNA was extracted from -80°C preserved liver samples of all treated and control groups [32]. Genomic DNA samples were electrophoresed on 1.5% agarose/ ethidium bromide gel for fragmentation assay [33].

Statistical Analysis: Data were subjected to statistical analysis including the calculation of the mean and standard error of the mean (SEM). Simple one way ANOVA was used and Duncan's Multiple Range test were used to differentiate between significant means [34].

RESULTS

Results of liver enzymes are presented in Table (1). You must sart the resuts of AST then ALT as you reported in Table 1. Alanine aminotransferase (ALT) activity significantly ($p<0.05$) decreased in atrazine and camphor treated animals (group2 and3) and also decreased in camphor and atrazine treated animals (group4) but this decrease was not significant. In contrast, aspartate aminotransferase (AST) activity increased in all treated animals compared to control but this increase is not significant.

Rats treated with camphor(group2) have insignificantly low serum testosterone concentration compared to control(group1) and camphor and atrazine treated rats(group 4) but those treated with atrazine(group3) have significantly high testosterone (Table 2). Camphor has the same effect on estradiol levels. While the estradiol (E2) concentrations were not significantly changed among different groups (Table 2), but rats treated with camphor and atrazine have high estradiol concentrations. Camphor increased the aromatizition of tesosterone to estradiol in atrazinand camphor group and consequently reduced tesosterone levels near to control levels. The aromatization of testosterone to estradiol was not changed in camphor and atrazine treated rats and is nearly similar to control rats. However, high testosterone to estradiol ratio is similar and high in both camphor and atrazine treated rats (Table 2).

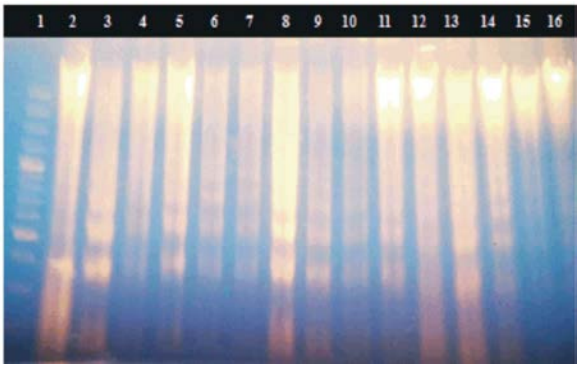

Fig. 1: DNA fragmentation analysis: 1.5% of agarose gel electrophoresis of DNA samples extracted from liver tissues of different groups of rats. Lane 1 represents 100 bp DNA marker, lanes from 2 to 5 represent DNA from liver of atrazine treated rats, lanes from 6 to 10 represent DNA from liver tissue of camphor treated rats, lanes from 11 to 15 represent DNA samples from liver tissues of atrazine+camphor treated rats, lane 16 represent DNA from liver of control rats.

Table 1: Effect of exposure of male rats of group 1, 2,3and4 on activities of AST and ALT

Animal groups	AST(U/L)	ALT(U/L)
Group1	78.15±6.63	74.13±5.13
Group2	88.39±10.29	55.22±5.56
Group3	105.49±8.48	55.53±4.96
Group4	108.19±8.51	62.71±4.33
P-Value	0.07	0.05

Means with different superscripts a, b are significant at $P<0.05$

Table 2: The levels of estradiol(E2), (pg/mL) and testosterone (pg/mL) and testosterone to estratiol ratio (Testo:E2) in rats group 1, 2,3and4

Animal goups	Testosterone (nmol/L)*	Estradiol (pg/ml)	Testo:E2
Group1	11.38 ± 1.29^{a}	13.27±2.21	0.99±0.31
Group2	9.30 ± 1.72^{a}	8.27±0.78	1.63±0.19
Group3	16.70 ± 0.69^{b}	11.50±2.09	1.62±0.22
Group4	11.31 ± 1.74^{a}	15.20±4.1	0.88±0.16
P-Value	0.01	0.07	0.09

Means with different superscripts a, b are significant different at $P<0.05$.

Table 3: levels of MDA, TAC and SOD in rats group 1, 2,3and4

Anial groups	MDA (nmol /ml)	TCA (mM/L)	SOD (U/ml)
Group1	9.52±0.68	0.74±0.01	363.06 ± 46.66^{a}
Group2	8.23±0.59	0.75±0.02	359.35 ± 43.67^{a}
Group3	9.17±1.22	0.75±0.01	385.38 ± 47.62^{a}
Group4	10.63±2.20	0.73±0.02	545.10 ± 40.67^{b}
P-Value	0.66	0.92	0.021

Means with different superscripts a, b are significant different at $P<0.05$

MDA and TAC levels are not significantly altered in all groups (Table 3). But the insignificant decrease in MDA levels suggests an antioxidant effect to camphor. However, a significant ($p<0.05$) increase in the activity of SOD in camphor and atrazine treated groups indicated that both camphor and atrazine increase oxidative stress. Also, a slight non-significant increase in SOD levels is observed in atrazine treated group.

***DNA* Fragmentation Analysis:** As shown in Figure (1), DNA of liver tissues from atrazine treated rats showed a high degree of fragmentation represented by ladder like shape (Lanes 2-5). DNA of liver from camphor treated rats shows highly smearing (Lanes 6-10), while DNA of camphorand atrazine treated rats shows ladder like shape fragmentation and high smearing of DNA (Lanes 11-15) in comparing with control one (Lane 16).

DISCUSSION

The measurement of blood ALT and AST released into the blood by damaged hepatocytes is a useful tool in the assessment of liver toxicity [35]. As a consequence, cellular damage to this tissue causes a substantial

increase in the blood levels of this enzyme, a fact rendering it an excellent marker of cellular necrosis. In agreement with our results, serum ALT activity is frequently associated with hepatotoxic effects but the latter is not always correlated with the histological findings [36]. In contrast, Campos-Pereira *et al*. [7] found discretely elevated ALT levels in the atrazine treated group when compared to the control group, but the difference was not significant. The significantly higher AST activities in animals exposed to atrazine compared with control groups may be due to the leakage of aminotransferase enzymes from injured liver cells [8]. On the contrary, rats treated with 400 mg/kg atrazine for 14 consecutive days had a non significant elevation in serum ALT enzyme [37]. SOD is an enzyme that contributes to the first line of antioxidant pathway as it removes oxygen radicals, repairs cells and reduces the damage done to them by superoxide, the most common free radical in the body [39]. In the present study SOD activities were significantly higher in the plasma of atrazine and camphor/atrazine treated groups. The decreased levels of MDA and SOD in camphor treated rats can be referred to that constituents of the *Cinnamomum camphora* had shown antioxidant activities [40]. Eugenol is the main constituent (About 81-85%) of *Cinnamomum* leaf oil [41] and it has been widely used in medical practice due to its antioxidant and anti-inflammatory properties [42]. Although camphor has insecticidal, antimicrobial, antiviral, anticoccidial, anti-nociceptive, anticancer and antitussive activities, in addition to its use as a skin penetration enhancer, but camphor is considered a very toxic substance [43]. As well as, administration of camphor influenced the levels of hepatic and extrahepatic reduced glutathione in mice [44]. This finding came compatible with other studies [8, 9]. In contrast to our results, Campos-Pereira *et al*. [7] found a significant increase of MDA levels and consequent lipid peroxidation indicative of plasma membrane damage. As well as, Nwani *et al*. [45] and Bhatti *et al*. [46] who demonstrated interaction of atrazine with biomembranes, but the slight non significant increase in MDA recorded in the present study in atrazine and camphor treated rats may refer to aggravations in the toxicity of atrazine dose when administered with camphor. The association between increased MDA levels and the activity of cytotoxic, genotoxic and oxidative stress-inducing products reported by Hidalgo and Zamora [47] is in agreement with the findings of Campos-Pereira *et al*. [7]. Similar to our results, the activities of SOD was not influenced by atrazine and its metabilote diaminochlorotriazine exposure in the serum of rats administered 200 mg/kg diaminochlorotriazine treated group compared with that of the control group [48].

Atrazine can also alter the activity of the hypothalamic-pituitary-adrenal (HPA) axis as indicated by the changes in circulating serum steroid concentrations. Dose dependent increases in progesterone release in both male [49] and female rats [50] have been observed following a single exposure to atrazine. Increased serum estrone and estradiol in male rats was observed following atrazine administration [4,14, 51]. In agreement with our results, both mass spectrometry and radioimmunoassay demonstrated similar results of increased levels of testosterone and estradiol (E2) of male rats exposed to atrazine (200 mg/kg) for five days with significantly increased serum concentrations of E2 (1.5-fold) and insignificant increased serum concentrations (1.3-fold) of testosterone [52]. In contrast to our results, male rats, exposed to atrazine from postnatal day 21 until 53 caused a significant decrease in serum and testicular testosterone levels when administered at doses of 100–200 mg/kg body weight [4].

Oral application of atrazine to peripubertal male rats for 28 days down regulated the expression of genes for steroidogenic enzymes and regulatory proteins involved in the control of testicular steroidogenesis [53]. Atrazine stimulates the expression of several genes responsible for steroidogenesis: SF-1, StAR, CYP17A1 and 17bHSD [54]. The insignificant change of estradiol concentrations mong different groups of rats of this study were inconsistence with that of Pogrmic-Majkic *et al*. [54].

In contrast to our results, the administration of atrazine and diaminochlorotriazine decreased the transcription levels of key genes related to cholesterol transport and testosterone (T) synthesis including scavenger receptor class B type 1 (SR-B1), cytochrome P450 cholesterol side chain cleavage enzyme (P450scc) and cytochrome P450 17-hydroxysteroid dehydrogenase ($P45017^2$) in testes [48]. Furthermore, the treatment of rats with 200 mg/kg diaminochlorotriazine significantly decreased the serum and testicular testosterone levels, while the treatment with 200 mg/kg atrazine significantly decreased the testicular testosterone levels [48]. In addition atrazine inhibits luteinizing hormone (LH) and testosterone production when applied at concentrations at or above 100 mg/kg per day and that reduction is secondary to weight loss [55]. Contrary to the results of this study, treatment with atrazine at a dose of 50 mg/kg bw/day reduced significantly the serum and intratesticular testosterone levels, both acutely from day 46 to 48 and

chronically from day 22 to 48 [10]. Cooper *et al*. [3] demonstrated that atrazine caused a dose- and time-dependent decrease in the amplitude of the LH surge and these endocrine changes appear to occur primarily by atrazine-induced changes in the hypothalamus. In addition, Stoker *et al*. [4] mentioned that atrazine exposure induced structural disruption in the testis of male rats. In contrast to our results, Friedmann [10] and Trentacoste *et al*. [55] reported reduced circulating levels of testosterone in atrazine exposed peripubertal male rats. Prolonged in vivo treatment with atrazine causes downregulation of testicular androgenesis [4, 10, 55].

A camphor derivative (3-benzylidene camphor; 3BC) is used in personal care products and in a number of materials for UV protection. 3BC has been shown in vitro and in vivo in fish to be estrogenic [56]. On the other hand, when three doses 4, 20, 40 µg/ 10µl of atrazine in alcohol were used to treat rats. Despite the central administration of camphor in hypothalamus - pituitary - gonad (HPG) axis, no significant differences were seen in sex hormones levels compared to the control. With this finding, it can be concluded that camphor may not effectively handle the axis via central pathway [57]. Several doses of Camphor affected all parts of the rat male reproductive system such as testis, seminal vesicles and vas deference [20].

Regarding to the effect of atrazine on DNA, our results showed that ATZ had the ability to induce significant elevation in the percentage of DNA fragmentation. This observation supported our previous study about genotoxicity of atrazine in mice [58] and also genotoxicity of ATZ by Singh *et al*. [59]. In agreement with our results, Cavas [60] found significant increases in the frequencies of micronuclei and DNA strand breaks in erythrocytes of *C. auratus,* following exposure to commercial formulation of ATZ and thus demonstrated the genotoxic potential of this pesticide on fish. Also significantly longer comet tails of DNA damage in leukocytes and isolated hepatocytes of male Japanese quail (*Coturnix japonica*) were recorded with 500 mg/kg bw ATZ [9]. In the same context, dominant lethal mutations in mouse spermatids and DNA strand breaks were observed in rat stomach, liver and kidney but not in rat lung, also after oral administration of high toxic doses [61, 62]. In contrast with our results, seventeen of the 23 gene mutation studies following in vitro exposure of mammalian cell lines to atrazine were negative; gene mutation studies on atrazine metabolites were also negative. Hence, governmental agencies responsible for reviewing and interpreting toxicology data have concluded that atrazine is not genotoxic [63-65]. In the same context, the tests with ATZ also evidenced a non genotoxicaction for mammalian cells in vivo and in vitro, although some positive results for tests of chromosomal aberrations and of DNA damage have been found in human lymphocytes, besides of DNA damage and formation of micronuclei in rats and mice [66]. In our study, using of camphor did not induce any protection against atrazine as the DNA samples from atrazine with camphor group showed extent of DNA damage and fragmentation (not less than that of atrazine group) which may predispose of cancer. In agreement with this result, it was also shown that continuous exposure of mice to camphor caused the appearance of cancer symptoms [67]. As well as, the study of cannamomum camphora leaves extract has shown the protective effects against DNA damage and biochemical changes in mice caused by atrazine. The results showed a significant and time-dependent decrease in the percentage of micronuclei, chromosomal aberrations and DNA damage in all tested tissues as compared to ATZ treated mice [58].

CONCLUSION

In conclusion, this study suggested that exposure to atrazine has negative effects on liver function as well as testosterone secretion in male rats. Also, atrazine induced high degree of DNA fragmentation of exposed heatic tissues. In addition, it provides evidence that camphor oil had no protective properties against exposure to atrazine.

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