PROTECTIVE EFFECT OF ELLAGIC ACID AGAINST CARBON TETRACHLORIDE (CCl₄) – INDUCED OXIDATIVE BRAIN INJURY IN RATS

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ABSTRACT

In this study, it was aimed to investigate protective effects of Ellagic acid in rats which have brain damage formed with carbon tetrachloride (CCl₄). 28 male Wistar albino rats were separated into 4 groups as Control, CCl₄, Ellagic acid and CCl₄+ Ellagic acid. From the brain tissue homogenate malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), glutathion peroxidase (GSH-Px), catalase (CAT) levels were measured and routine histopathological investigation was performed. An increase in MDA level (p<0.01) whereas a decrease in CAT, GSH-Px, SOD (p<0.01) and GSH (p<0.05) levels in CCl₄ administered group compared to control was observed. In our study, in the control and Ellagic acid administered groups, no microscopic findings were observed in the brain, while severe lesions were seen in the CCl₄ administered group and only mild congestion lesions were seen in the CCl₄ + Ellagic acid group. Results of this study suggest a protection by ellagic acid against CCl₄ induced brain damage. This protection is possibly via induction of antioxidant protective mechanism which is shown both by biochemical and histopathological methods.

KEYWORDS:
Brain damage, Carbon tetrachloride, Ellagic Acid, Rat

INTRODUCTION

Reactive oxygen species are among the etiology of degenerative diseases. Exposure to radiation, heavy metals and xenobiotics cause increase in free radicals and oxidative stress [1,2]. Carbon tetrachloride (CCl₄) poisoning resembles oxidative stress as an experimental model [3]. CCl₄ exerts toxic effects in various organs such as kidney, testis, heart, lung, brain and mainly on liver [4]. CCl₄ toxicity probably is due to formation of trichloromethyl radical. In the presence of oxygen, CCl₄ reacts with oxygen to form trichloromethyl peroxide radical [5,6]. Following CCl₄ toxification, augmented oxidative stress due to increased free radical formation is thought to be the reason behind tissue damage [7]. Ellagic acid is a polyphenolic compound found in pomegranate, mulberry, walnut, green tea, strawberry and eucalyptus leaf [8,9]. Ellagic acid have different mechanisms of action in bioactivity studies but most proven ones are antioxidant, anti-fibrotic, anti-carcinogenic, antiplasmodial activity and chemoprotective property [10,11]. Such properties of ellagic acid are thought to be beneficial against organ damage. Therefore this study was aimed to investigate protective effect of ellagic acid against CCl₄ induced acute brain damage in rats.

MATERIALS AND METHODS

Animals. This study was conducted in Bingol University with the approval of Bingol University Animal Experiments Local Ethical Commission (04.04.2017-2017/04.04.01). 28 male Wistar-Albino rats weighing 250-300 gr were used. Rats were kept in a room with 20-22°C constant temperature within twelve (12h) hours of light-dark cycle (lights on 07:00-19:00; lights off 19:00-07:00). Rats were given water and standard pellet food ad libitum and acclimatized to their cages for adaptation. Only feeding was ceased 12 hours prior to experiments.

Experimental Design. Rats were divided into four groups containing 7 animals each; Group I (Control, n=7): Animals in this group was injected with distilled water, 250-300 grams of body weight daily for 7 days [12]. Group II (CCl₄, n=7): Administered 0.5 ml/kg CCl₄ (i.p.) daily for 7 days [12]. Group III (Ellagic acid, n=7): Administered ellagic acid (5 mg/kg/day) (oral gauge) daily for 7 days [13]. Group IV (CCl₄+ Ellagic acid, n=7): Administered 0.5 ml/kg CCl₄ (i.p.) daily for 7 days + ellagic acid 5 mg/kg/day with oral gauge. Brain tissue samples were obtained from all groups at the...
end of 7th day under i.p. 60 mg/kg ketamine hydrochloride and 10 mg/kg Xylasine anesthesia. Brain tissue was obtained, washed with saline, dried and stored in deep freeze (-80℃) biochemical (MDA, GSH, SOD, GSH-Px, CAT) analysis were conducted.

**BIOCHEMICAL ASSAYS**

**Measurement of MDA Level.** MDA levels in tissues were determined spectrophotometrically with method of Ohkawa et al. 532 nm concentration was given as nmol/mg protein [14].

**Measurement of GSH Level.** Tissue GSH levels were determined with the method defined by Tietze (1969) and Fairbans et al. (1999). 405 nm concentration was given as μmol/mg protein [15, 16].

**Measurement of SOD Level.** SOD (EC 1.15.1.1) activity in the tissue was determined by at 505 nm wavelength according to using method of Sun et al. (1988). This method relies on Xanthine oxidase induced production of O2:radical and avoidance of nitroblue tetrazolium (NBT) reduction with the help of SOD enzyme. [17]. The results were expressed as IU/mg protein in terms of specific activity.

**Measurement of GSH-Px Level.** Activity of GSH-Px (E.C.1.11.1.9) was determined by using Paglia et al. (1967). Decrease of absorbance via oxidation of NADPH to NADP during such reactions was read with Microplate Reader at 340 nm GSH-Px activity was measured [18]. The results were expressed as IU/mg protein in terms of specific activity.

**Measurement of CAT Level.** CAT (EC 1.11.1.6) activity was measured according to the method described by Aebi 1974. H2O2 gives maximum absorbance in 240 nm wavelength. It relies on disintegration of H2O2 by catalase into water and oxygen and it is observed as the decrease in absorbance in UV spectrometer. Decrease in absorbance is related with catalase enzyme activity. Decrease of absorbance with sample addition was read in every 15 second for 5 min. For calculation values of every minute linear absorbance decrease is evaluated [19]. The results were expressed as IU/mg protein in terms of specific activity.

**Histopathological analysis of the brain.** After treatment, eight rats in each group were anaesthetized with diethyl ether, euthanized by decapitation under deep anesthesia and necropsied. After that the cerebrum were excised and isolated immediately. Also, brain tissue samples were fixed in 10% neutral-buffered formalin for 48 h. The tissue samples were processed as routine protocol of dehydration with alcohol series (50%, 70%, 96% and 100%), clearance in xylene and then embed in paraffin at 5 μm thickness with rotary microtome (Leica, RM2125) [20]. The slides were stained with hematoxylin and eosin for histological examination [21]. Finally the slides were examined and photographed using light microscopy (Leica, DM2500).

**Statistical analysis.** Results were evaluated with SPSS 20(Statistical Program- Software System) program. Data were expressed as X ± SEM. One way ANOVA was conducted and intragroup comparisons were done with post-hoc Tukey test.

**RESULTS**

MDA levels were found higher in CCl4 administered group (CCl4) compared to control group (Cont) (p<0.01). In addition, increase in CCl4 + Ellagic Acid administered group was significant (p<0.05) and also decrease in CCl4 + Ellagic Acid compared to lone CCl4 administered group was found significant (p<0.01) (Figure 1) GSH was found significantly lower in CCl4 and in CCl4 + Ellagic Acid group compared to control (p<0.05) (Figure 2). Decrease in SOD values in CCl4 and CCl4 + Ellagic acid groups compared to control group was found statistically significant (p<0.01). In addition increase in SOD level in CCl4 + Ellagic acid group compared to CCl4 group was found significant (p<0.05). (Figure 3), Increase in GSH-Px level in Ellagic acid and CCl4 + Ellagic Acid compared to CCl4 group were found significantly higher (p<0.05) (Figure 4), CAT enzyme activity levels were found significantly lower in CCl4 + Ellagic acid group compared to control (p<0.01). In addition, this level was found significantly higher in Ellagic acid compared to CCl4 group (p<0.05). Also increase in CCl4 + Ellagic acid was significantly higher compared to CCl4 group (p<0.01) (Figure 5). In control group (Group-1) no significant histopathological differences were noted and the brain tissues were in normal histological architecture (Figure 6- A). In the CCl4 administered group (Group-2) in brain, perineuronal vacuolation, neuronophagia, mild congestion of blood vessels and glial cell proliferation around damaged neuronal cell bodies to give rise to so-called satellitosis were found most frequently in cerebral cortex. Common lesion in the cerebral cortex are as follows; necrosis of most of pyramidal neurons with pyknotic nuclei, neuronal shrinkage and mild microgliosis (Figure 6- B). In addition, no significant histopathological evidences were observed after administration of Ellagic acid group (Group-3) in the section of rat brain, which is similar to control group (Figure 6- C). Only mild congestion lesion of blood vessels in cortex has been observed in CCl4 + Ellagic acid group (Group-4) (Figure 6 - D).
FIGURE 1
MDA levels (nmol/mg protein) of brain tissues of different groups b (*), b1(***)=p<0.01, c(**)=p<0.05

FIGURE 2
GSH levels (μmol/mg protein) of brain tissues in different groups b (*), b1(***)=p<0.01, c(**), c1(****)=p<0.05

FIGURE 3
SOD levels (IU/mg protein) of brain tissues of groups b (*), b1(***)=p<0.01, c(**)=p<0.05
FIGURE 4
GSH-Px levels (IU/mg protein) in brain tissues of groups c(**), c1(****)=p<0.05

FIGURE 5
CAT levels (IU/mg protein) in brain tissues of groups b (*), b1(***)=p<0.01, c(**), c1(****)=p<0.05

FIGURE 6
Histopathology of brain tissues. (A): Control group (Group-1), normal histological architecture x10. (B): CCl₄ administered group (Group-2), perineural vacuolation, neuronophagia and satellitosis (arrows); necrosis of pyramidal neurons with pyknotic nuclei and neuronal shrinkage (arrow heads) x20. (C): Ellagic acid group (Group-3), normal histological structure x10. (D): CCl₄ + Ellagic acid group (Group-4), mild congestion of blood vessels in cortex (arrows) x10. H&E staining.
**DISCUSSION**

CCL<sub>4</sub> is a toxic agent which causes free radical formation due to its toxic effect [22]. CCL<sub>4</sub> is transformed by cytochrome P450 enzyme into trichloromethyl (CCL<sub>3</sub>) and trichloromethyl peroxide (CCL<sub>3</sub>OO·) free radicals which are more toxic. Following their production lipid peroxidation occurs which causes tissue oxidative damage [23-26]. Oxidative stress is the disruption of the balance between free radicals and antioxidant defense system which may be caused by a pathological event. Organisms are protected against oxidative stress via enzymatic and non-enzymatic systems [27]. In many of the previous studies with CCL<sub>4</sub>, it was shown that antioxidant defense systems are involved in protection of the animals against this toxication [28, 29]. MDA is among substances formed by lipid peroxidation and a commonly used parameter as an indicator for oxidative stress [30]. In our study CCL<sub>4</sub> injected group had a significant rise in brain MDA levels compared to control group. Increase of MDA proves augmentation of lipid peroxidation in brain and oxidative stress as an outcome in brain due to CCL<sub>4</sub>. Similarly it is reported that MDA levels in the brains of CCL<sub>4</sub> administered animals rise [2]. CCL<sub>4</sub> + Ellagic acid administered group showed a decrease in MDA level due to Ellagic acid (p<0.05) (Figure 1). This decrease may be due to amelioration of the oxidative damage in the brain caused by free radicals by ellagic acid. Literature reports support our data in this aspect. Ellagic acid was also tested on STZ induced diabetics in rats in literature and ellagic acid protected brain and sciatic nerve against lipid peroxidation [31]. In a study focusing on cisplatin induced oxidative stress on heart and liver showed protective effect of Ellagic acid [32]. GSH involves in certain steps in defense processes against oxidative damage caused by free radicals, peroxides and other toxic substances and protects cells [33]. Therefore decrease of GSH makes cells more prone to such hazards. Attenuation of GSH in living organisms leads to tissue malformations and injuries [34]. In our study CCL<sub>4</sub> injected group showed significant decrease in GSH level compared to control however ellagic acid alleviated this decrease to some extent (p<0.05). (Figure 2). This protection might be related with antioxidant nature of ellagic acid. Recent studies provided evidence for protective effect of ellagic acid against oxidative stress. Literature studies present decrease of GSH in 6<sup>th</sup> hour due to CCL<sub>4</sub> [35] or in heart and liver of rats due to cisplatin [32]. Mentioned decreases in GSH levels might be due to overuse of GSH for decreasing toxic effects of CCL<sub>4</sub>. SOD is an important enzyme which scavenges peroxide anion radicals and inhibits its lipid peroxidation due to free radicals [36]. Brain tissue SOD activity was significantly lower in CCL<sub>4</sub> and CCL<sub>4</sub> + Ellagic acid injected group compared to control (p<0.01) (Figure 3). Similar studies present an important decrease in brain tissue SOD activity in CCL<sub>4</sub> administered groups [2, 4, 37]. Reason for this decrease was explained as induction of hydrogen peroxide and peroxynitrite formation in brain tissue by CCL<sub>4</sub> and decrease of such enzymes during removal of such reactive substances from tissue. Literature studies are in accordance with our findings. SOD levels significantly increased in CCL<sub>4</sub> + Ellagic acid administered group compared to lone CCL<sub>4</sub> injected group (p<0.05) (Figure 3). In a study focusing on hepatoprotective effect of ellagic acid in rats, SOD activity was found lower in CCL<sub>4</sub> injected group compared to CCL<sub>4</sub> + Ellagic acid administered group [38]. Literature findings support our results in this parameter. GSH-Px plays an important role in cell defense against reactive oxygen species. GSH-Px levels in our study significantly increased in CCL<sub>4</sub> + Ellagic acid administered group compared to lone CCL<sub>4</sub> administered group (p<0.05) (Figure 4). Other researchers found an increase in GSH-Px activity in CCL<sub>4</sub> + Ellagic acid administered group compared to lone CCL<sub>4</sub> injected group in a study focusing on hepatoprotective activity of ellagic acid in rats [38]. CAT is among important enzymes which avoids free radical accumulation and lipid peroxidation [39]. In our study although CAT was decreased in CCL<sub>4</sub> + Ellagic acid group compared to control (p<0.01), increase in ellagic acid compared to CCL<sub>4</sub> group was also found significant (p<0.05). In addition increase in CCL<sub>4</sub> + Ellagic acid group compared to CCL<sub>4</sub> group was also found significant (p<0.01) (Figure 5). In literature CCL<sub>4</sub> administered group showed a significant decrease in brain tissue CAT activity [2, 4, 37]. Similarly decrease in kidney tissue CAT activity was reported in CCL<sub>4</sub> administered rats [40]. Literature reports also support our findings. In our study, difference between the groups was observed in histopathological findings in the brains as a result of microscopic examination. In the control and Ellagic acid administered groups, no microscopic findings were observed in the brain, while severe lesions were seen in the CCL<sub>4</sub> administered group and only mild congestion lesions were seen in the CCL<sub>4</sub> + Ellagic acid group. Although administration of CCL<sub>4</sub> was reported to cause severe necrosis and hemorrhage at the meninges in the rat brain, it has not been observed in our study. Histopathological findings which are observed in rat brain tissues due to CCL<sub>4</sub> toxicities, CCL<sub>4</sub> administered group (Group-2) such as perineural vacuolation, neuronophagia, mild congestion of blood vessels and satellitosis, necrosis of most of pyramidal neurons with pyknotic nuclei, neuronal shrinkage and mild microgliosis found most frequently in cerebral cortex, are similar to some CCL<sub>4</sub> toxicity studies [2, 41]. Ellagic acid administration was reported for its neuroprotective activity by decreasing neuronal damage and necrosis in streptozotocin-induced diabetic rat
brains [31]. In our study, it might be proposed that Ellagic acid has neuroprotective effect against CCl4 induced oxidative rat brain damage in CCl4 + Ellagic acid group (Group-4) similar to their control group and our findings (Figure 6).

CONCLUSION

Results of this study suggest a protection by ellagic acid against CCl4 induced brain damage. This protection is possibly via induction of antioxidant protective mechanism which is shown both by biochemical and histopathological methods.

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Authors declare no conflict of interest.

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