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Neuroprotective effects of Zingerone against carbon tetrachloride (CCl_4) induced brain mitochondrial toxicity in Swiss albino mice

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Abstract
The present study targeted the brain mitochondria dysfunction in Swiss albino mice through carbon tetrachloride intoxication and its treatment with Zingerone. It is proposed that brain mitochondria is the main organelle responsible for oxidative stress by producing reactive oxygen species (ROS). Swiss albino mice were divided into four groups; Group-1 was control; Group-2 was carbon tetrachloride (CCl_4) toxic (1.5mg kg^{-1} bm i.p two days in a week.); Group-3 was pretreated with Zingerone (100 mg kg^{-1} b.m) a day before the administration of CCl_4 and Group-4 was only Zingerone (100 mg kg^{-1} bm) given orally for 15days once in a day. At the end of the experiment mice were sacrificed and mitochondria were isolated from brain. Isolated brain mitochondria were further analyzed for oxidative stress marker. Thiobarbituric acid reactive substance (TBARS) content was increased significantly by CCl_4 administration in Group-II as compared to the control Group-I, while the antioxidant (GSH) and other antioxidant enzyme GPx , GR, and CAT was depleted significantly in CCl_4 treated Group-II as compare to control Group -I. Zingerone protected the toxicity of brain mitochondria by reducing the lipid peroxidation and enhancing the antioxidant enzyme in Group-III and there was no significant changes were noticed in Group-IV as compared to Group-I. Overall results showed the potential effects of Zingerone in protecting the neuronal cell loss by oxidative stress. Thus, the present study indicated that the Zingerone may be used as the potential therapeutic tools for the prevention of CCl_4 induced brain mitochondrial toxicity.

Keywords: Brain Mitochondria, Carbon tetrachloride, Oxidative stress, Zingerone

INTRODUCTION

Mitochondria are often referred to as the powerhouse of the cells and it is found in every cell in our bodies. They generate the chemical energy in the form of adenosine tri phosphate (ATP) that regulates fueling of cellular processes. Brain cells need a lot of energy to communicate and control the cell signaling with each other that indicate the importance of mitochondria for normal brain function. The deterioration of mitochondria or dysfunction of mitochondrial energy metabolism in the brain cells is a primary cause of all neurodegenerative disorders due to generation of reactive oxygen species (ROS) (Cassarino and Bennette, 1999). Carbon tetrachloride (CCl_4) is known clear, colorless and nonflammable liquid repeatedly used in scientific research for induce of free radical in different tissues of animals (Alam, 2017; Sahreen, et al., 2011; Khan et al., 2011; Ritesh et al., 2015). CCl_4 produced trichloromethyl free radical after the biotransformation through chain reaction process (Adewole, 2012,).
Several plants (*Withania somnifera*, *Azadirachta indica*, *Aerva lanata*, *Coriandrum sativum* and *Nigella sativa*) have been reported as high medicinal value including antioxidant properites (Attanayake and Jayatilaka, 2016). Zingerone is one of the active constituent isolated from *Zingiber officinale* rhizome and having high antioxidant properties (Ahmad, et al 2015). Several reports are available of Zingerone in management of oxidative stress in different tissues (Aeschbach, et al 1994). Recently Alam et al. (2017) has reported the Therapeutic action mechanism of Zingerone against CCl_4 induced liver mitochondrial toxicity in Swiss albino mice. Zingerone has also been reported to inhibit the lipid peroxidation and enhances the antioxidant enzyme in brain tissue (Mohammed, 2015). Zingerone is quickly metabolized in rat and easily crosses the blood brain barrier and reaches in the systemic circulation and is eliminated from the body within 6hrs after oral administration (Huang, 2010, Zick et al, 2008). Brain is rich in polyunsaturated fatty acids and it is much prone to oxidative stress. Brain mitochondria can play an important role in oxidative stress mechanism. Therefore, the present study targeted the brain mitochondria dysfunction through carbon tetrachloride intoxication and its treatment

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with Zingerone.

MATERIALS AND METHODS

Drug and chemicals: Zingerone, 1-chloro-2,4-dinitrobenzene (CDNB), 5-5′-dithio-bis-2-nitrobenzoicacid (DTNB), Thiobarbituric acid (TBA), Glutathione (oxidized Sigma-Aldrich, tinamide adenine dinucleotide phosphate reduced form (NADPH), Hydrogen peroxide, Sulfosalisylic acid, Sucrose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Ethylene diamine tetracetic acid (EDTA), Percoll and other chemicals with reagent grade etc. used for the experiments were of Sigma-Aldrich , USA.

Experimental model: Male Swiss albino mice (35 -50 g) were taken from Central Animal House of College of Pharmacy, Jazan University. Mice were acclimatized in laboratory at maintaining standard condition (12h light/dark cycle) for animals. Male mice were divided into four groups: Group-1 was control and vehicle was given orally and Group-II was experimental and CCl_4 at a dose of (1:1 v/v) solution in olive oil/paraffin oil) 1.5 mg kg^{-1} bm i.p. twice a week for 15 days. Group-III were pretreated with Zingerone 100 $mgkg^{-1}$ bm for 15 days orally) and followed by CCl_4 at a dose of 1.5 mg kg^{-1} bm i.p. Group-IV was given only Zingerone (100 $mgkg^{-1}$ bm) from the starting of the experiments. Each group had six animals.

Mitochondria isolation: At the end of experiment, mice were sacrificed, brain were isolated and further brain mitochondria were isolated as described by Mohammed M Safhi (2015). In detail, 10% homogenate (w/v) of brain tissue was prepared in isolation buffer I (0.32 M sucrose; 5.0 mM HEPES, pH 7.5; 0.1 mM EDTA) at 1000 rpm in homogenizer. After homogenization of tissue, it was centrifuged at 1000g for 10 min at 4°C and cell debris (pellet 1) was discarded and supernatant was taken (S1). The supernatant (S1) was again centrifuged at 12,500g for 20 min at 4°C and pellet 2 was used for further mitochondrial isolation and supernatant was discarded. Pure mitochondria were isolated in isolation buffer II (0.25 M sucrose, 5.0 mM HEPES, pH 7.2, 0.1 mM EDTA-K+) on Percoll gradients of 8.5%, 10%, and 20% and centrifuged at 15,000g for 20 min at 4°C. The bottom layer were used as mg/ml protein after resuspension in isolation buffer.

Lipid peroxidation test: Lipid peroxidation test was estimated by the method of Utley et al. (1967) in the form of Thiobarbituric acid reactive substance (TBARS). In brief 0.1 ml homogenate was incubated at 37°C in ametabolic shaker and another equal volume of homogenate wasplaced at 0°C for 1 h. After the incubation, 0.5ml of 5%(w/v) chilled TCA and 0.5 ml of 0.67% TBA were added and centrifuged at 4000×g for 10min. Thus, supernatant was collected in test tubes and placed in a boiling water bath for 10min. After that the pink colour was produced and it was measured at 535 nm in a spectrophotometer (Shimadzu-1601, Japan). The TBARS content was calculated by using a molarextinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nanomoles of TBARS formed per hourmg^{-1} of protein.

Glutathione test: Glutathion test (GSH) was estimated by the method of Jollow et al. (1974). In brief, sample was mixed with 4% sulfosalisylic acid (w/v) in 1:1 ratio (v/v). The samples were incubated at 4°C for 1 h, and centrifuged at 4000×g for 10 min at 4°C. Further, assay mixture was added 1.0mM DTNB and the yellow color was read at 412 nm in a spectrophotometer (Shimadzu-1601, Japan). The GSH content was calculated as micromoles GSH mg^{-1} protein, using a molarextinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

Antioxidant enzyme (GPx, GR, and CAT) Test: Glutathione peroxidase (GPx) was estimated by Mohandas et al.(1984) procedure. The enzyme activity was calculated as nmol (NADPH) oxidized/min/mgprotein by using molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$. Glutathione reductase (GR) activity was measured by the methodof Carlberg and Mannervik (1975) as modified by Mohandas et al. (1984). The enzyme activity was quantified at room temperature by measuring the disappearance of NADPH at 340 nm and calculated as nmol NADPH oxidized/min/mg protein usingmolar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}. Catalase (CAT) activity was measured by the procedure of Claiborne A, (1985). The change in absorbance was recorded at 240 nm. Catalase activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein using molarextinction coefficient of 43.6×10^3 M^{-1} cm^{-1}.

Protein test: Protein was estimated by the method of Lowry et al.(1975) using bovine serum albumin as standard on 660nm.

Statistical analysis: Results are expressed as mean ± SEM. The result wasanalyzed by one-way ANOVA followed by Turkey's test. The p value ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

The Zingerone effect on lipid peroxidation (TBARS content) was measured to estimate the membranous damage by oxidative stress. It was noticed that TBARS content in brain mitochondria was elevated significantly ($p<0.001$) in carbon tetrachloride induced group (Group-II) as compared to control group (Group-I) and it was protected significantly ($p<0.01$) by Zingerone administration in Group-III as compared to Group-II (Fig.1). There was no changes observed after administration of Zingerone (100mg kg^{-1}) in Group –IV as compared to Group-I. The glutathione (GSH) level was significantly ($p<0.01$) decreased in CCl_4 induced brain mitochondrial toxicity in Group-II as compared to Control Group-I. Pre-

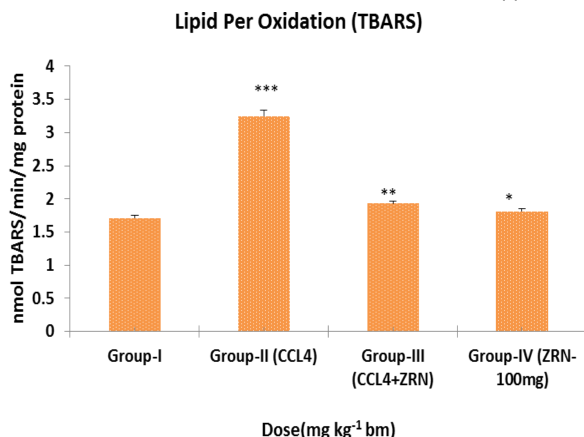

Fig.1. *Effects Carbon Tetrachloride on TBARS content in brain Mitochondria of Swiss albino mice and Treatment with Zingerone (ZRN). *p<0.01vs. Group-I, **p<0.01vs. Group-II, ***p<0.001 Group-II vs. Group-I*

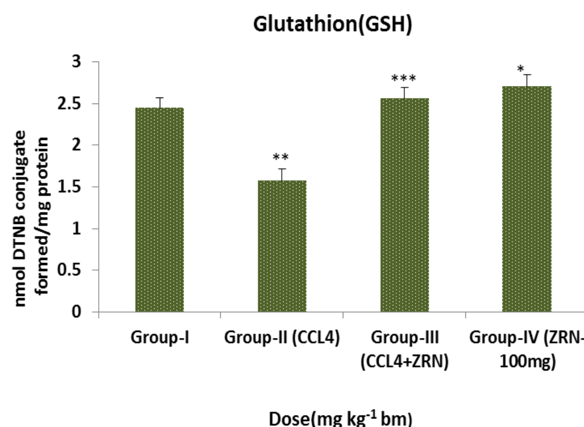

Fig.2. *Effects Carbon Tetrachloride on GSH content in brain Mitochondria of Swiss albino mice and Treatment with Zingerone (ZRN). *p<0.01vs. Group-I, **p<0.01vs. Group-I, ***p<0.001 Group-III vs. Group-II.*

treatment with Zingerone has protected the reduced glutathione (GSH) level significantly (P<0.001) and dose dependently manner in Group-III as compared to Group-II. (Fig.2). There was no significant changes were observed after administration of Zingerone (100mg kg^{-1}) in Group –IV as compared to Group-I. The administration of CCl_4 significantly depleted the antioxidant enzymes (GPx, GR, and CAT) in Group-II as compared to Group-I in brain mitochondria of Swiss albino mice. While the administration of Zingerone protected the antioxidant enzyme significantly in Group-III when compared with Group-II. There was no changes observed in Group –IV as compared to Group-I (Table 1).

Brain is the important organ with high concentration of lipids and is very much susceptible to reactive oxygen species (ROS) (Freeman 1982). Mitochondria are one of the sites for production of ROS in brain that is easily targeted by neurotoxin. Thus neurotoxin such as carbon tetrachloride ameliriate the oxidative stress in brain mitochondria which may leads to several neurological diseases. Oluwafemi *et al.,* 2016 have been recently reported that CCl_4 induced oxidative stress in rat brain. De Souza *et al.,* 2015 have also been reported that CCl_4 increases the pro inflammatory cytokine level in rat brain. Thus, carbon tetrachloride is a neurotoxin which easily cross the blood brain barrier and leads to generation of free radicals that ultimately develop oxidative stress in the brain. Glutathione is a tripeptide (γ-L-glutamyl-L-cysteinylglycine) is found in the highest concentration in all cells that is also called body's master antioxidant. It helps in the neutralization of free radicals and reactive oxygen compounds, (Scholz et al., 1989, Safhi M, 2015). In this study it was noticed that GSH level was depleted significantly (P<0.01) in Group-II, which was also significantly (P<0.001) restored in Zingerone pretreatment group Group-III.

Glutathione peroxidase (GPx) is selenium containing antioxidant enzyme that effectively degrade the hydrogen peroxide and lipid peroxides to water. Thus lipid alcohol and in turn oxidized glutathione to glutathione disulphide. In absence of adequate GPx activity or glutathione levels hydrogen peroxide and lipid peroxides are not detoxified and may be converted to OH-radical and peroxyl radicals respectively. Reduced glutathione(GSH) is regenerated from the GSSG by glutathione reductase (GR) enzyme with presence of nicotinamide ade-

Table 1. Neuroprotective effect of Zingerone on antioxidant enzymes in brain mitochondria.

Groups	GPx (nmol of NADPH oxidized/min/mg protein)	GR (nmol of NADPH oxidized/min/mg protein)	CAT (nmol of H_2O_2 consumed/min/mg protein)
Group-I (Control)	582.21±20.11	665.30±30.11	361.21±26.42
Group-II (CCl4)	315.17±31.73**	403.33±28..41*	151.37±31.46*
Group-III (CCl4+Zingerone)	619.43±94.81$^{\#\#}$	587.64±31.21$^{\#}$	345.07±44.11$^{\#\#}$
Group-IV (Zingerone)	597.10±31.8$^{\$}$	695.70±28.13$^{\$\$}$	373.21±25.31$^{\$}$

Values are expressed as Mean±SEM. (*p<0.01, **p<0.01, Group-II vs.Group-I). ($^{\#}$p<0.05, $^{\#\#}$p<0.01 Group-III vs. Group-II). (($^{\$}$p<0.05, $^{\$\$}$p<0.01 Group-IV vs. Group-I).

nine dinucleotide phosphate (NADPH) (Warsy, 1999). Thus, GPx and GR are important antioxidant enzyme which help to maintain the cellular redoxcyle. Catalase is usually located in a cellular, bipolar environment organelle called the peroxisome (Alberts et al., 2002). It catalyzes the decomposition of hydrogen peroxide into water and oxygen.

The above all these antioxidant enzymes were decreased significantly and confirming the role of oxidative stress by the administration of CCl_4 in brain mitochondria of mice and it was also significantly managed by the administration of Zingerone. Zingerone is known for potential pharmacological action such as antioxidant properties which play an important role to manage the oxidative stress in brain. Zingerone quickly metabolize in rat and human and easily cross the blood brain barrier that help in the manage of oxidative stress in rat brain (Zick et al., 2008 and Huang, 2010). Zingerone protected the Parkinson's disease in mice against 6-hydroxydopamine (Kabuto H, et al, 2005). Safhi Mohammed M (2015) reported that Zingerone protects the Tellurium toxicity in the brain mitochondria of rats. Alam M. F et. al (2017) also reported that the Therapeutic action mechanism of Zingerone against CCl_4 induced liver mitochondrial toxicity in Swiss albino mice.

Conclusion

Thus, the above study indicates that the effectiveness of Zingerone against CCl_4 induced brain mitochondrial toxicity in mice. It can easily manage the brain mitochondrial toxicity in mice. As we know that Zingerone is a part of our daily diet which may be used as good therapeutic potential for the prevention of CCl_4 induced toxicity in brain mitochondria. Therefore, it is further need to explore the other mechanism such as molecular aspect of neuro protection in mice.

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