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Research Article

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Fisetin Modulates the $CoCl_2$ Induced Oxidative Stress in Cerebral Cortex of Mice Brain

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ABSTRACT

Oxidative stress is common mechanism during hypoxia that resulted to molecular alterations such as lipid peroxidation in cerebral cortex of mouse brain. Oxidative stress during hypoxia is due to depletion of antioxidant enzymes and reduced glutathione. Oxidative stress during hypoxia in cerebral cortex is due to depletion of antioxidant enzymes. Fisetin, a dietary flavonol, protects brain cells against oxidative stress in vitro. In addition, fisetin protects hepatocytes against oxidative stress by modulating the activity of antioxidant enzymes. Thus, It has been hypothesize that fisetin could protect cerebral cortex against oxidative stress in vivo. Aim of this study was to evaluate the antioxidant efficacy of fisetin against $CoCl_2$-hypoxia in the mouse cerebral cortex in vivo. Fifteen days oral administration of $CoCl_2$ to mice resulted in a significant increase in levels of reactive oxygen species (ROS) without altering serum aspartat transaminase (AST) and alanine transaminase (ALT), indicating no damage to liver. Increased levels of ROS resulted to increased level of lipid peroxidation. In addition decreased levels of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) activity and non-enzymatic antioxidant GSH further confirmed the $CoCl_2$ induced oxidative stress in cerebral cortex of mice brain. Continuous treatment with fisetin (5 mg/kg) orally twice daily for 15 days significantly restore the antioxidant enzymes and GSH in cerebral cortex resulted to reduced level of ROS and LPO and offered almost complete protection. Fisetin has potential antioxidant against oxidative stress induced by $CoCl_2$-hypoxia in the cerebral cortex of the mice brain.

Keywords: $CoCl_2$; Hypoxia; Oxidative stress; Reactive oxygen species; Fisetin; Cerebral cortex

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Abbreviations:

AST: Aspartat Transaminase; ALT: Alanine Transaminase; SOD: Superoxide Dismutase; CAT: Catalase; GPx: Glutathine Peroxidase; LPO: Lipid Peroxidation

INTRODUCTION

High metabolic activity of brain requires an uninterrupted supply of oxygen to maintain high metabolic activities [1-3]. Hypoxia, a condition in which brain gets less oxygen than it requires. Accumulation of reactive oxygen species (ROS) during hypoxia resulted to molecular alterations [4]. ROS impart molecular alterations such as lipid peroxidation, DNA damage and protein alterations which induce to neuronal death and neural dysfunction [5]. ROS are short lived molecule thus it mostly impart molecular alteration at the site of production. Lipids are most

preferred molecule that under go ROS induced alterations. Cellular ROS comprises superoxide anion, hydrogen peroxide, and hydroxyl radical: consist of radical and non-radical oxygen species formed by the partial reduction of oxygen. This accumulation of ROS during hypoxia is mainly due to depleted level of enzymatic and non-enzymatic antioxidants [6-8]. In addition hypoxia has been reported to affect a variety of physiological process such as cognitive decline by affecting long term potentiation (LTP), adaptation to high altitudes, erythropoiesis and cancer [6,9-13].

ROS accumulation during hypoxia induces lipid per-oxidation that resulted to altered brain functions [14]. ROS is autonomously detoxified by superoxide dismutase (SOD) in the cell. SOD catalyzes the O_2- (ROS) into H_2O_2. Activity of SOD is conserved since evolution of Archaea bacteria. This is most critical enzyme which allows the cells to detoxify the ROS in oxygen rich environment. Inhibition of SOD with 2-methoxyestradiol was demonstrated to induce apoptosis in the leukemia cells through a free radical-mediated mechanism. It has been reported that $CoCl_2$ induced hypoxia also causes oxidative stress by lowering the activity of antioxidant enzymes such as superoxide dismutase (SOD), Catalase (CAT) [7,8]. In addition it has been reported that hypoxia also declined the level of GSH *in vitro* [5]. Thus, Cobalt chloride ($CoCl_2$) is widely used model in both *in vivo* and *in vitro* studies to generate artificial hypoxia like conditions by stabilizing HIF1α [15]. Hypoxia inducible factor does so by modulating the transcription of SOD and Catalase [8].

Fisetin, a dietary natural flavonol, is found in vegetables and fruits. Various therapeutic roles of flavonols have been reported on various models. Fisetin promoted neurite growth in PC12 cells [16]. Further Fisetin restore cognitive decline by modulating long term potentiation (LTP) and it also affect small clot metabolism in brain [13]. Fisetin, structure containing hydroxyl groups suggest its potential antioxidant property. In silico studies on fisetin suggested its potential *in vitro* antioxidant property. Further, this in silico study is supported by bond dissociation energy of hydroxyl bond and dipole movement [17]. Fisetin strongly bind between polar head and hydrophobic tail of phospholipid and this region is most proximate to serve as antioxidant against lipid peroxidation. Thus, Fisetin could protect against ROS at the site of production. Most important is fisetin increases the level of GSH in HT22 cells, PC12 cell line *in vitro* [18]. Fisetin has been reported to increase haemoxygenase-1 (HO-1) level in endothelial cells [19]. Fisetin has been reported to increase the level of antioxidant enzymes SOD, CAT and GPx in liver *in vivo* and kidney and glutathione *in vitro* [6,20].

$CoCl_2$ induced hypoxia causes oxidative stress by lowering the activity of antioxidant enzymes such as SOD, CAT, GPx and Glutathione level leading to impairment of normal brain function. Most of the *in vivo* studies on mainly on liver and kidney suggested that fisetin could able to modulate the activity of antioxidant enzymes and fisetin also increased the level of GSH in hippocample HT22 cell line *in vitro*. In addition in silico studies suggest that fisetin could fit among the phospholipid thus provide local protection against oxidative stress. Therefore, present study was carried out with the objective of evaluating the efficacy of dietary flavonoid fisetin in maintaining the balance in the oxidant-antioxidant status during $CoCl_2$ induced hypoxia induced oxidative stress in cerebral cortex of mice brain.

MATERIALS AND METHODS

Chemicals

$CoCl_2$, DMSO and fisetin were obtained from Sigma-Chemical Co. (St. Louis, MO, USA). Other reagents were of analytical grade obtained from SRL. All solutions were prepared with ultra-pure water purified by a Milli-Q Gradient system. $CoCl_2$ solution was prepared in warm PBS.

Animals

Swiss albino male mice weighing 25-30 g were procured from central animal house Banaras Hindu University, Varanasi, India. The mice were acclimatized for a week before start of experiment. They are kept at temperature 22 ± 2℃ with relative humidity at 65 ± 10% and at the photoperiod of 12 h light dark cycle. Standard rodent diet and water were provided the animals ad libitum. All the experiments were carried out according to the guideline of Institutional animal ethics Committee.

Experimental Design

Mice were divided into four groups, with six mice in each group. The control group received a single oral dose of saline solution and 20 µL of DMSO (Group 1). The Fisetin group was given only Fisetin dissolved in 20 µL of DMSO per mouse 5 mg/kg body weight, twice a day until sacrifice mixed in 0.50 mL PBS. $CoCl_2$ group 40 mg/Kg (dissolved in 0.5 mL PBS/mouse) body wt till sacrificed. The combination group (Fisetin + $CoCl_2$) received $CoCl_2$ 40 mg/Kg body wt followed by two doses of fisetin (5 mg/kg body weight, i.p.) twice a day until sacrifice. Animals were sacrificed 15 days after the treatment schedule. Mice were anesthetized with sodium pentobarbitone

immediately before sacrifice and sacrificed by decapitation cerebral cortex were dissected out and washed with 0.9% NaCl immediately brain extract was prepared.

Preparation of Brain Homogenate

Cerebral cortex was homogenized in buffer (0.2 M Tris-Cl (pH 7.4) containing protease inhibitors. Extract were centrifuged at 35000 x g for 45 min at 4˚C. Supernatant were decanted out in respective tubes and kept on ice. Protein content was determined by Lowery method [21]. Studies on antioxidant and other biochemical assay were completed using the freshly prepared homogenate.

Estimation of ROS Level

NBT reduction assay was performed as reported previously following the method of Maurya et al. [6]. In freshly prepared 1% homogenate in PBS, NBT-PBS solution was added in ratio of 1:1 incubated at 37 for 4 h. The mixture was mixed gently and centrifuged. The pellet obtained was washed three times and dissolve in 1 mL of each KOH (2 M) and DMSO. Optical density was recorded at 630 nm and compared with standard plot constructed against NBT. Values were expressed as μ mole of NBT/mg protein.

Estimation of Lipid Peroxidation

Briefly using the method [22] product of lipid peroxidation is malondialdehyde (MDA). 0.5 mL of the brain extract was mixed with 0.5 mL of 0.2 M Tris-maleate buffer (pH 5.9) and mixture was incubated on water bath at 37˚C for 30 min. 1.5 mL of thiobarbituric acid (TBA) was added in the mixture, was incubated in boiling water bath for 10 min using tight condenser. After the mixture was cool down 4 mL of 1 N NaOH were added and allowed to stand for 10 min. OD was recorded at 548 nm and level of lipid peroxidation were expressed as nmol MDA/mg protein.

SOD Activity Assay

SOD activity was estimated by the method of Kakar et al. [23]. Reaction 109 mixture containing 0.1 mL of phenazine methosulphate (186 μmol), 1.2 mL of sodium pyrophosphate buffer (0.052 mmol; pH 7.0) 0.3 mL of Brain extract was added. Assay started with addition of 0.2 mL of NADPH (780 μmol) and stopped after 1 min by adding 1 mL Glacial acetic acid. OD was recorded at 560 nm. Results were expressed as unit/mg protein.

Catalase Activity Assay

Catalase activity was measured in the brain extract following the method of Bergmeyer et al. [24] with some modifications. In brief, in 2 mL suitably diluted brain extract 1 mL substrate solution (0.75 ml of 30% H_2O_2 in 100 mL of 0.05 M phosphate buffer, pH 7.0, 25°C) was added. Decrease in absorbance was recorded at 240 nm for 3 min against 0.05 M potassium phosphate buffer (pH 7.0) as blank. The change in absorbance/min was converted into unit of the enzyme and degradation of 1 μmol of H_2O_2 per min at 25˚C was defined as one unit of catalase taking extinction coefficient of H_2O_2. Values were presented as unit/mg protein.

GPx Activity Assay

Glutathione peroxidase was assayed following the method as used by Singh [25] In 1 mL assay buffer (100 mM Tris-HCl (pH 7.2), 3 mM EDTA, 1 mM sodium azide, 0.25 mM H_2O_2, 0.5 mM NADPH, 0.17 mM GSH, and one unit of glutathione reductase) freshly prepared brain extract containing 40 μL protein was added. Reduction in OD was recorded at 340 nm for 4 min. One unit of GPx was defined as 1 μmol of NADH produced per min at 25˚C. Activity is expressed as unit/mg of protein.

GSH Level

Total glutathione was determined by using the method with slite modifications [6] as described. Briefly for total glutathione, 0.2 mL of Brain extract was precipitated with 0.100 mL of 5% SSA (Sulfosalic acid) and after centrifugation supernatant was neutralized. 0.05 mL of supernatant was incubated with 0.10 mL of reagent (0.30 mM NADPH, 0.22 mM DTNB, 1 mM EDTA, 1.6 units/mL GR prepared in 100 mM phosphate buffer, pH 7.4) absorbance was recorded at 412 nm for 10 min.

Statistical Analysis

All data were expressed as mean ± SD Statistical analyses were conducted by unpaired Student's t-test and. The level of significance between saline control and Fisetin treated group was set at @<0.05, Fisetin (F) and CoCl2

group #p<0.05, ##p<.01, ###p<0.001 and $CoCl_2$ and Fisetin-combined group *p<0.05, **p<.01, ***p<0.001 in all sets.

RESULTS

Optimization of $CoCl_2$ dose to Induce Oxidative Stress in Brain Without Liver Damage

In order to achieve optimum $CoCl_2$ dose which induce oxidative stress without damaging liver; various doses of $CoCl_2$ was administered orally to mice of all batches and the level of ROS in cerebral cortex and LFT parameters in liver were examined. Findings revealed that $CoCl_2$ administration gradually increased the ROS level upto 60 mg/Kg body wt in cerebral cortex however higher dose of $CoCl_2$ administration also showed significant change in LFT parameters. Thus data suggested that 40 mg per Kg body wt. could be optimum dose for the study. Moreover preliminary data also suggested that after 10 days treatment with $CoCl_2$ significant changes were observed in ROS level at higher dose but an optimum level of ROS observed in 40 mg/Kg body wt after 15 days. Thus we started treatment of fisetin after 10 days of $CoCl_2$ administration which continued up to 15 days. Therefore, the 40 mg/kg BW dose of $CoCl_2$ was used for further experiments. More over 10 mg/Kg body wt dose of Fisetin twice a day was used for treatment as standardize in our lab and previous report [6].

Fisetin declined the level of ROS in cerebral cortex $CoCl_2$ induced oxidative stress is main cause of alteration in brain function. To ensure whether Fisetin could decline the ROS level in cerebral cortex ROS level between control and treated group were compared. A significant rise of ROS level was observed in cerebral cortex of $CoCl_2$ group as compared to control groups. However after fisetin treatment, enhanced level of ROS declined to regain its normal value.

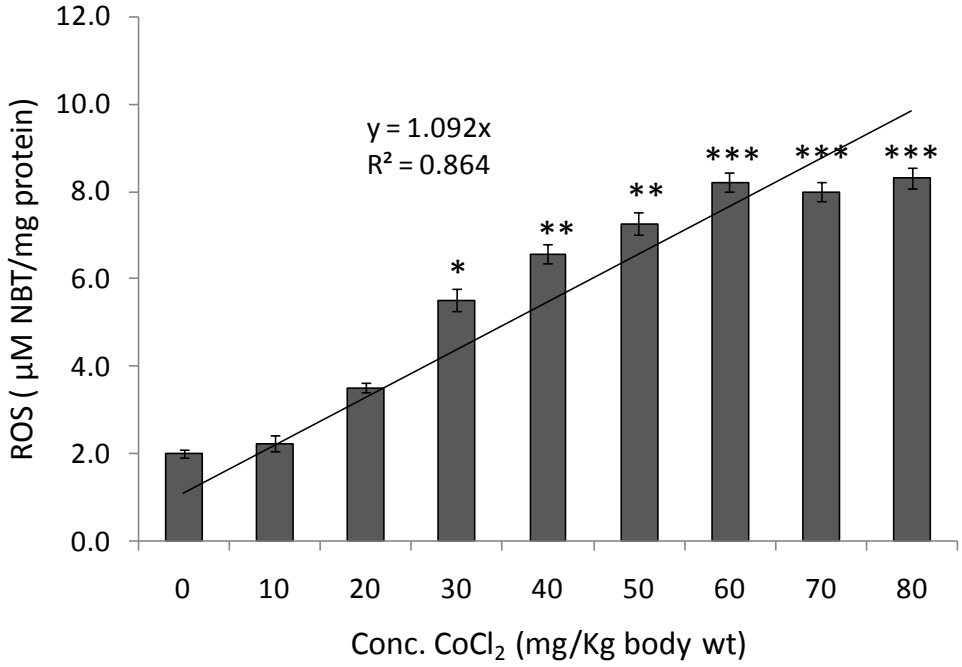

Figure 1: Effect of $CoCl_2$ induced hypoxia on ROS level in cerebral cortex of mice

Results are given as mean ± SD for six mice. Comparisons are made between: control mice and $CoCl_2$ administered group with different doses of $CoCl_2$ and level of significance * p<0.05, ** p<0.001, *** p<0.001.

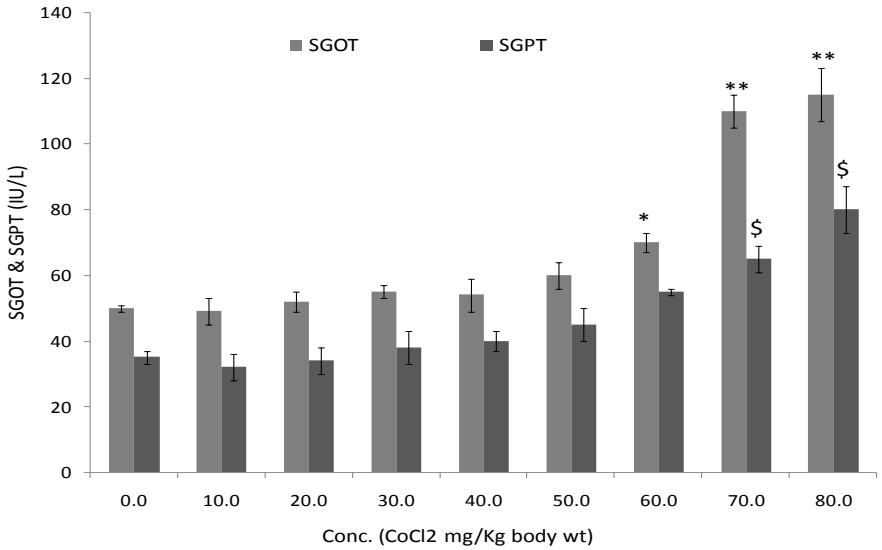

Figure 2: Effect of $CoCl_2$ induced hypoxia on SGOT and SGPT level in blood serum of mice administered with different doses of $CoCl_2$

Results are given as mean ± SD. for six mice. Comparisons are made between: control mice and $CoCl_2$ administered group with different doses of $CoCl_2$ and level of significance * $p<0.05$, ** $p<0.001$, *** $p<0.001$ (SGOT) and $ $p<0.05$.

Fisetin Declined the Level of ROS and MDA in Cerebral Cortex

ROS induced one of the spontaneous site of alteration is lipid peroxidation. To ensure whether Fisetin could decline the MDA level in cerebral cortex, MDA level between control and treated group were compared. A significant rise of MDA level was observed in cerebral cortex of $CoCl_2$ group as compared to control groups. However after Fisetin treatment, enhanced level of MDA declined to regain its normal value.

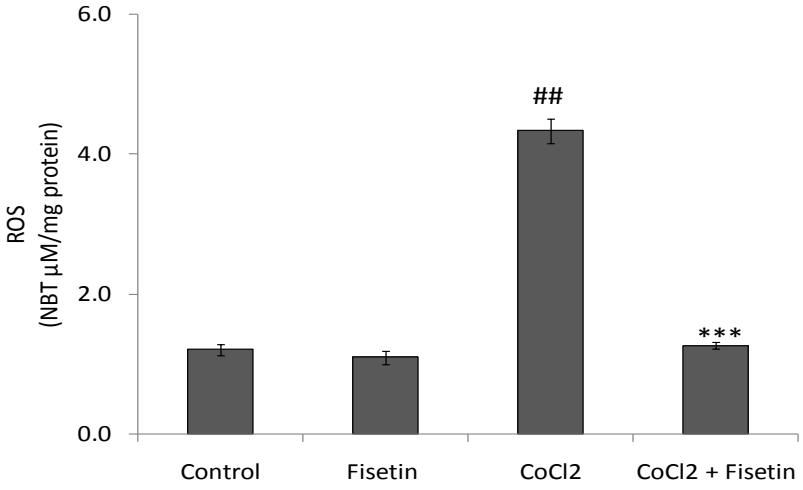

Figure 3: Effect of Fisetin on ROS level in cerebral cortex tissue of experimental animals during $CoCl_2$ induced hypoxia

Results are given as mean ± S.E.M. for six rats. Comparisons are made between: control mice (Group I); Fisetin treated mice (Group II), Control mice (Group I); $CoCl_2$ treated mice (Group III) and $CoCl_2$ (Group III) and $CoCl_2$-Fisetin combined group (Group IV). *$p<0.05$, ** $p<0.001$, *** $p<0.001$.

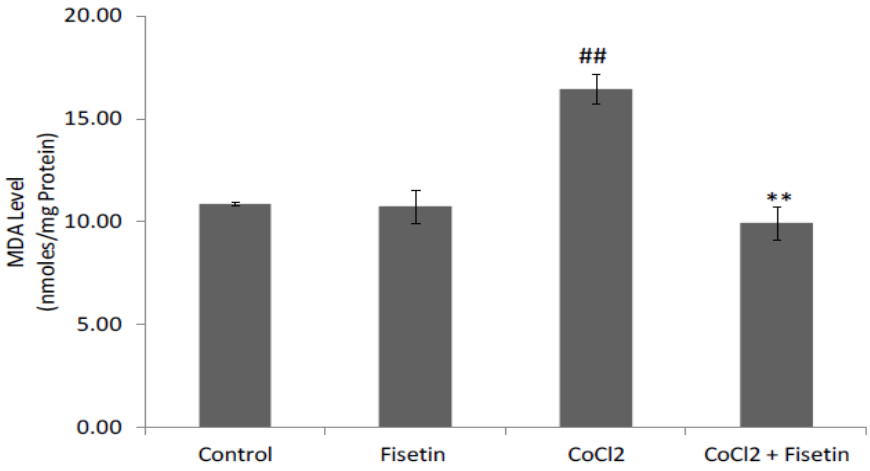

Figure 4: Effect of Fisetin on MDA level in cerebral cortex tissue of experimental animals during $CoCl_2$ induced hypoxia

Results are given as mean ± S.E.M. for six rats. Comparisons are made between: control mice (Group I); Fisetin treated mice (Group II), Control mice (Group I); $CoCl_2$ treated mice (Group III) and $CoCl_2$ (Group III) and $CoCl_2$-Fisetin combined group (Group IV). *p<0.05, ** p<0.001, *** p<0.001.

Effect of Fisetin on Antioxidant Enzymes: SOD, CAT and GPx

Declined level of SOD1 is accountable for Oxidative stress in cerebral cortex. In present study we compared the SOD1 activity in control cerebral cotex and two experimental group mice. Significant declined activity was observed in $CoCl_2$ group as compared to controls. Fisetin treatment could able to regain the SOD1 activity at normal level. SOD1 is committed enzyme that neutralizes the free radicals to hydrogen peroxide. Catalase is fastest enzyme in cellular system which metabolizes hydrogen peroxide. Thus, Catalase plays an important role in hydrogen peroxide metabolism. Active level of CAT found to be declined in $CoCl_2$ group as compared to controls in cerebral cortex. However, after fisetin treatment activity of CAT enhanced significantly and regained its normal value in cerebral cortex. GPx is another enzyme which metabolizes hydrogen peroxide using GSH as substrate. A similar pattern of GPx activity was observed like CAT activity.

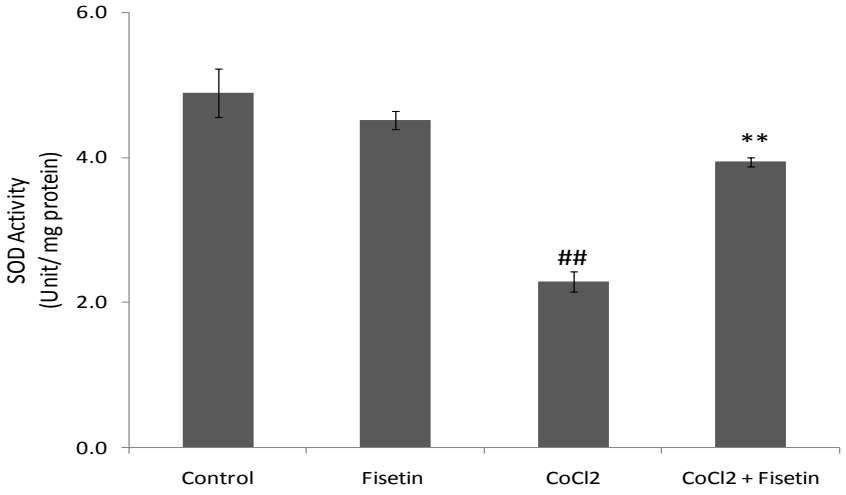

Figure 5: Effect of Fisetin on SOD activity in cerebral cortex tissue of experimental animals during CoCl2 induced hypoxia

Results are given as mean ± S.E.M. for six mice. Comparisons are made between: control mice (Group I); Fisetin treated mice (Group II), Control mice (Group I); $CoCl_2$ treated mice (Group III) and $CoCl_2$ (Group III) and $CoCl_2$-Fisetin combined group (Group IV). *p<0.05, ** p<0.001, *** p<0.001.

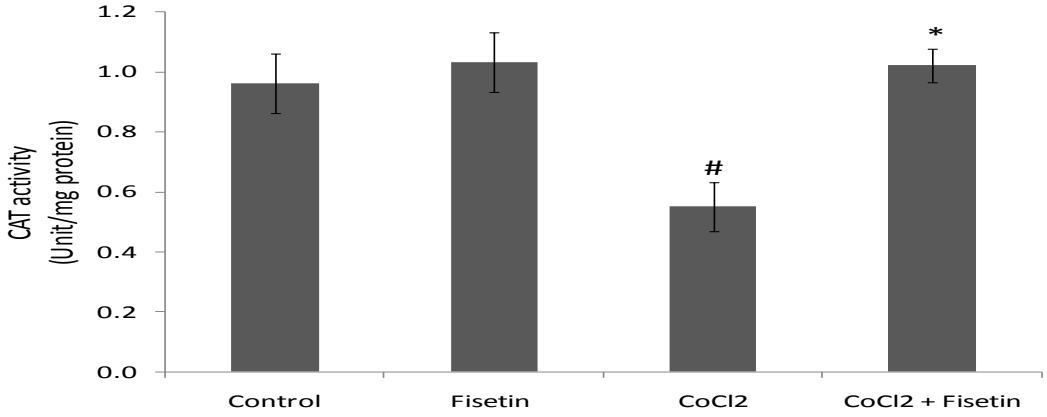

Figure 6: Effect of Fisetin on CAT activity in cerebral cortex tissue of experimental animals during $CoCl_2$ induced hypoxia

Results are given as mean ± S.E.M. for six mice. Comparisons are made between: control mice (Group I); Fisetin treated mice (Group II), Control mice (Group I); $CoCl_2$ treated mice (Group III) and $CoCl_2$ (Group III) and $CoCl_2$-Fisetin combined group (Group IV). *p<0.05, ** p<0.001, *** p<0.001.

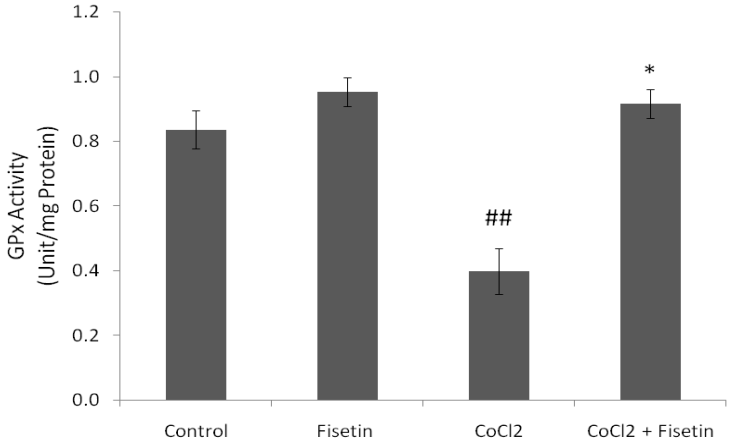

Figure 7: Effect of fisetin on GPx activity in cerebral cortex tissue of experimental animals during CoCl2 induced hypoxia

Results are given as mean ± S.E.M. for six mice. Comparisons are made between: control mice (Group I); Fisetin treated mice (Group II), Control mice (Group I); $CoCl_2$ treated mice (Group III) and $CoCl_2$ (Group III) and $CoCl_2$-Fisetin combined group (Group IV). *p<0.05, ** p<0.001, *** p<0.001.

Effect of Fisetin on Antioxidant Enzymes: Glutathione (GSH) Level

GSH is one of the important quencher of hydrogen peroxide. We observed a significant increased level of GSH in fisetin treated group as compared to control and significantly declined level of GSH was observed in $CoCl_2$ group fisetin treatment significantly increased the level of GSH in combined group.

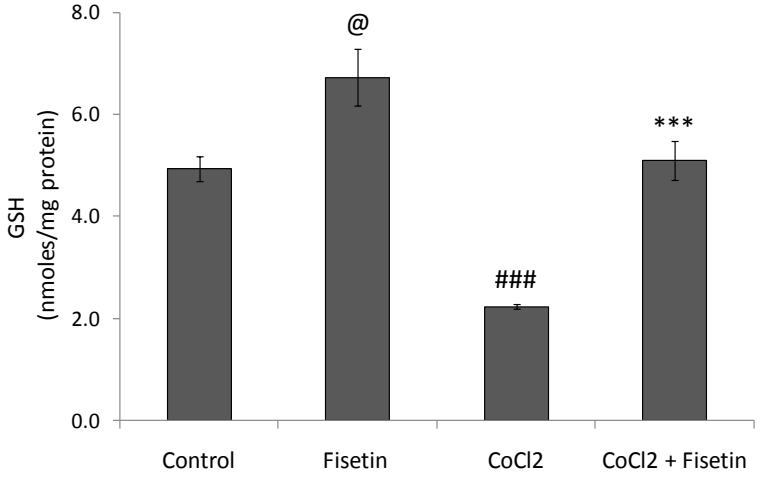

Figure 8: Effect of Fisetin on GSH level in cerebral cortex tissue of experimental animals during $CoCl_2$ induced hypoxia

Results are given as mean ± S.E.M. for six mice. Comparisons are made between: control mice (Group I); Fisetin treated mice (Group II) *p<0.05, Control mice (Group I); $CoCl_2$ treated mice (Group III) and $CoCl_2$ (Group III) and $CoCl_2$- Fisetin combined group (Group IV). *p<0.05, ** p<0.001, *** p<0.001.

DISCUSSION

Hypoxia induced lipid peroxidation is due to accumulation of ROS [4]. To generate artificial hypoxia like conditions, $CoCl_2$ has been used in both *in vivo* and *in vitro* studies [8,15]. But to optimize a $CoCl_2$ dose that induces oxidative stress in cerebral cortex without damaging liver is critical before proceeding to the actual experiment. In order to achieve optimum $CoCl_2$ dose, various doses of $CoCl_2$ was administered orally to mice of all batches and the level of ROS in cerebral cortex and LFT parameters in liver were examined (Figures 1 and 2). >40 mg/Kg body wt. dose of $CoCl_2$ induced marked elevation of ALT and AST in serum which is a marker of liver damage [26,27]. Moreover preliminary data also suggested that after 10 days treatment with $CoCl_2$ significant changes were observed in ROS level at>40 mg/Kg body wt. But 40 mg/Kg body wt dose for 15 days was found most suitable for the study. A significant rise of ROS level was observed in cerebral cortex of $CoCl_2$ group as compared to control groups (Figure 3). Our study confirms the oxidative stress in $CoCl_2$ induced oxidative stress in mice cerebral cortex. 40 mg/Kg body wt. is most suitable dose to induce oxidative stress in cerebral cortex. Our data also similar to the $CoCl_2$ dose reported [8].

Four hydroxyl groups in fisetin suggested its potential antioxidant activity. In addition, potential antioxidant property of fisetin has been reported earlier by in silico modeling *in vitro* and *in vivo* studies [13,17,18]. When antioxidant capability of the cell is compromised the oxidative damage in a cell or tissue occurs due to generation of relatively higher concentration of ROS. Several studies have also reported the similar effect of decline of ROS level in liver, kidney and *in vitro* studies on hippocampal HT22 [6,18,20]. Declined the ROS level $CoCl_2$ combined group further confirmed the antioxidant capability of fisetin.

Lipid peroxidation is one of the spontaneous alterations that ROS imparts that resulted to neuronal death. Status of lipid peroxidation is taken as direct evidence of oxidative stress [28]. In silico studies suggested the potential protective effect of fisetin against lipid peroxidation [17] thus immediate consequence to be studied for the alteration is lipid peroxidation. A significant rise of lipid peroxidation (MDA) level was observed in cerebral cortex of $CoCl_2$ group (Figure 4). Increased lipid peroxidation in $CoCl_2$ group corroborates with increases level of ROS. Fisetin treatment declined the LPO level in $CoCl_2$ group. Further suggest the potential antioxidant activity of fisetin. Several studies have also reported the similar effect of decline of MDA level in liver, kidney [6,20].

Accumulation of ROS is mainly due to depleted level of enzymatic and non enzymatic antioxidants [6,7]. SOD, CAT and GPx enzymes and GSH constitute the antioxidant mechanism against ROS [6]. $CoCl_2$ induced hypoxia declined the level of antioxidant enzymes SOD and Catalase [7,8]. Moreover, fisetin has been reported to increase the level of antioxidant enzymes SOD, CAT *in vivo* in liver [25]. Since, SOD is only enzyme that autonomously catalyses ROS into H_2O_2. Significant declined SOD activity was observed in $CoCl_2$ group [8] and we also observed the similar effect. Fisetin treatment could able to regain the SOD1 activity at normal level (Figure 5). SOD1 is committed enzyme that neutralizes the free radicals to hydrogen peroxide. Catalase is fastest enzyme in cellular system which metabolizes H_2O_2 into O_2 [29]. Thus, Catalase plays an important role in H_2O_2 metabolism. Active level of CAT found to be declined in $CoCl_2$ group as compared to controls in cerebral cortex. However, after fisetin treatment activity of CAT enhanced significantly and regained its normal value in cerebral cortex (Figures 6-8). Similar effect is also reported the in liver [6]. Similarly GPx is another enzyme which metabolizes hydrogen peroxide using GSH as substrate. A similar pattern of GPx activity was observed like CAT activity. Most important is Fisetin increases the level of GSH in hippocampal HT22 cells [18]. GSH is one of the important quencher of hydrogen peroxide. We observed a significant increased level of GSH in fisetin treated group as compared to control and significantly declined level of GSH was observed in $CoCl_2$ group. Fisetin treatment significantly increased the level of GSH in combined group. We found similar result as reported in liver [6].

Summary of Effect of Fisetin on Antioxidants in Cerebral Cortex

Fisetin modulated the GSH level and restores the antioxidant enzyme activity in cerebral cortex that gives protection against hypoxia induced oxidative stress. Consequence of this is protection of LPO in cerebral cortex.

CONCLUSION

Natural product could play critical role in treatment of drug induced toxicity [30]. However, mechanism of action of these products needs to be determined. Protective effect of fisetin against ROS has been reported at cellular level in liver [6] but brain is more susceptible to oxidative stress than liver. Fisetin restore GSH level thereby given protection against hypoxia induced oxidative stress. Effect of fisetin on GSH need to be explored further because our study indicates the fisetin might affect the synthesis of GSH [31,32]. Major limitation of this study is that we cannot able to separately present the antioxidant effect of fisetin and its effect on GSH synthesis. Fisetin modulated the activity of antioxidant enzymes and Glutathione to modulate the ROS level in cerebral cortex.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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