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Research Article

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Modulation of mGSH by Fisetin Protects Mitochondria against Cisplatin-Induced Oxidative Stress

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ABSTRACT

Reduced glutathione (GSH) is an important and abundant non-enzymatic antioxidant in the mitochondria. GSH pool in the mitochondria is maintained by the GSH transport from cytosol and recycling of the oxidized glutathione (GSSG) to GSH by NADPH dependent enzymes. GSH quench the reactive oxygen species (ROS) produced in mitochondria. CP is a widely used anti-cancer drug, high dose of it induces hepatotoxicity. CP induced hepatotoxicity is mainly due to depletion of GSH in the mitochondria which in turn induces ROS-LPO mediated mitochondrial damage. Fisetin, a dietary flavonol, found in fruits and vegetables, has been reported to protect kidney and liver against ROS by modulating the antioxidant enzymes of cytosol. In addition it has been reported that fisetin also up regulated the level of GSH in vitro. No report available on modulation of GSH level by fisetin in mitochondria in vivo. Therefore, we hypothesized that modulation of mitochondrial GSH (mGSH) level by fisetin could be a protective mechanism against the CP induced oxidative damage in mitochondria. Single dose of fisetin (100 mg/Kg body wt.) was evaluated against single dose of CP (6 mg/Kg body wt.) in vivo in mice taking appropriate controls. After experiment biochemical parameters were evaluated in the isolated liver mitochondria. Cisplatin induced depletion of GSH thereby upregulated the ROS level. Consequence of it ROS mediated lipid peroxidation (MDA level) and caspase 3 activation in cytosol. Fisetin restored the GSH level upto normal and thereby decreased level of ROS and ROS mediated consequences.

Keywords: Mitochondria; Lipid peroxidation; Partial reduction; Hepatotoxicity

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INTRODUCTION

Mitochondrion is major site of Reactive oxygen species (ROS) production. ROS produced in mitochondria are five to ten folds higher than cytosol [1]. Normally oxygen is fully reduced to water in mitochondria but, partial reduction of oxygen leads to production of superoxide anion (oxygen free radical) [2]. If, ROS are not restricted to threshold level it can damage the macromolecules such as lipid, protein and DNA. CP is a widely used anti-cancer drug, high dose of it induces hepatotoxicity [3]. Major cause of high dose of CP induce hepatotoxicity is mitochondrial oxidative stress [4]. Cause of CP induced oxidative stress conditions in mitochondria, is depletion of mGSH in matrix [5]. Reports on CP suggest that CP induced hepatotoxicity model is widely used to study mitochondrial dysfunction and apoptosis.

Antioxidants are important component of cell. Antioxidants are broadly categorized into two categories viz. enzymatic and non-enzymatic components. Mn-dependent superoxide dismutase (MnSOD), catalyze conversion of ROS into hydrogen peroxide in mitochondria [2]. The Glutathione peroxidase (Gpx4), enzyme associated with the mitochondrial inner membrane can directly and efficiently metabolize H_2O_2 [6]. Gpx activity protects mitochondrial membrane lipids against peroxides. As result of Gpx activity GSH is converted to GSSG, and H_2O_2 is neutralized. NADPH-dependent glutathione reductase convert back GSSG to GSH. Thus, GSH/GSSG ratio is an important

indicator of oxidative stress in the mitochondrial matrix [2]. Therefore, mGSH level is critical regulate the activity of GPx and GR in the mitochondria.

Among non-enzymatic components GSH is the most abundant component [7]. GSH is constitutively synthesized in the cytosol [8,9]. GSH is transported to mitochondria and other cellular compartments [7]. mGSH is important to maintain reducing environment in the mitochondrial matrix [10,11]. mGSH is maintained mainly via transport of GSH to mitochondria from cytosol [10,11]. Mitochondria contains very little <15% of total GSH in the cell but its level is critical to cell viability during mitochondrial oxidative stress [12,13]. mGSH is one of the most important determinants of response to oxidative stress. It assists to remove ROS by involving enzymatic and non enzymatic components. In addition, mitochondrial reducing pool is critical to mitochondrial function. Mitochondrial proteins constitutively undergo specific glutathionylation easily because pKa value of GSH is 8.3 and pH of mitochondrial matrix is slightly basic (pH~7.8) [14]. Oxidative stress conditions changes the GSH reducing to GSSG oxidizing environment in mitochondrial matrix. This, GSSG pool reduces the complex IV activity of electron transport chain that resulted to mitochondrial dysfunction [15]. This may slowdown the process of recycling of GSSG back to GSH by NADPH dependent enzymes. Thus, in oxidative stress conditions mitochondria critically depends on GSH transport from cytosol. Depletion of GSH level in cytosol spontaneously hampers the transport of GSH. Therefore, mGSH level depends on GSH level in cytosol and transport of it to mitochondria.

Apoptosis, also known as programmed cell death that is characterized by series of biochemical events [16]. Apoptosis that initiates due to mitochondrial dysfunction or damage is called as intrinsic pathway. Apoptosis or necrosis ultimately leads to activation of first initiator caspases (i.e., Caspase-2, 8, 9, and 10) thereby cascadic activation of executer caspases (i.e., Caspase-3, 6, 7) [16]. Depletion of mGSH predisposes lipids to oxidative modification by ROS and that could compromise the vital mitochondrial function [17]. In mitochondria, Cardiolipin, anionic phospholipid only found in mitochondria, involved in mitochondrial function and cell death regulation [18]. Cardiolipin found mostly on mitochondrial inner membrane associated with cytochome c [18]. It has been postulated that peroxidation of cardiolipin favor the release of cytochrome c [18,19]. Release of cytochrome c in cytosol induces apoptotic mechanism thereby caspase activation. In addition, TNF induced hepatocyte death and liver injury is through stimulation of mitochondrial ROS [20]. Depletion of mGSH is critical TNF induced mitochondrial membrane permialization, cyt. c release and caspase activation [21]. Therefore, activation of caspase 3 activity in cytosol may be an indicator of mitochondrial damage.

Fisetin, a natural falavonol, present in vegetables and fruits [22]. Studies revealed that fisetin administration could protect liver and kidney tissue against oxidative stress [23,24]. Most of the studies suggest that protective effect of fisetin is due to increased activity of antioxidant enzymes and cytosolic GSH [23]. Whether fisetin could modulate mGSH; reports are limited. Present study it has been hypothesized that fisetin administration may protect the CP induced mitochondrial oxidative damage via increasing mGSH level and increase the activity of antioxidant enzymes in mitochondria. Therefore, Fisetin administration may protect liver mitochondria against CP induced oxidative stress.

EXPERIMENTAL PROCEDURE

Animals

Swiss albino male mice weighing 25-30g 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°C with relative humidity at 65 ± 10% and at the photoperiod of 12h 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 animal ethics Committee.

Chemicals

CP and fisetin were obtained from Reddy's Lab (Hyderabad, India) and Sigma chemicals Co. (St. Louis, MO, USA) respectively. Rests of the chemicals were obtained from SRL, India.

Treatment Schedule

Animals were divided into four groups (n=6), a total of 24 male mice were used. Fisetin (100 mg/Kg b.w) was dissolved in corn oil. Cisplatin (6 mg/Kg b.w.) was obtained as dissolve formulation. The dose of CP was based on previous reports.

GROUP I: Normal Saline only (Orally)

GROUP II: Fisetin (100 mg/Kg b.w., orally) only

GROUP III: Fisetin (100 mg/Kg b.w., orally), 6 h before the administration of cisplatin (6 mg/Kg b.w. ip)
GROUP IV: Cisplatin (6 mg/Kg b.w.)
At the end of experiment (after 24 h after administration of CP) mice were sacrificed by cervical decapitation. The liver was immediately dissected out and washed with ice cold saline to remove blood. The effect of fisetin on cisplatin induced oxidative stress were studied using suitable biochemical parameters in the mitochondria.

Preparation of Mitochondrial Fraction
Fresh liver was washed with ice-cold saline solution and minced. Then, 10% homogenate was prepared in ice-cold medium (containing 0.2% BSA, 1mM EGTA, 250 mM sucrose, 10 mM Hepes-KOH, pH 7.4) using homogeneizer (Remi, India) using the method already reported [25]. Liver mitochondria were isolated by standard differential centrifugation method. The homogenate was centrifuged at 600×*g* for 5 min and the resulting supernatant was centrifuged at 15,000×*g* for 10 min. The pellet was resuspended in 10 mL of medium (Containing 0.1 mM EGTA, 0.12% BSA, 25 0mM sucrose, 10 mM Hepes- KOH, pH 7.4) and centrifuged at 15000×*g* for 10 min. All centrifugation procedures were performed at 4°C. The mitochondrial pellet obtained was resuspended in 1 mL medium (Containing 160 mM KCl and 10 mM Hepes-KOH, pH7.4). Mitochondrial suspension was kept on ice and used within 2 h. Protein concentration was determined by the Lowry method with BSA as a standard [26].

Measurement of ROS Level
NBT reduction assay was performed as reported [25]. Briefly, 1% liver homogenate, prepared in PBS, was added with the NBT-PBS (1mg NBT/mL) solution in the ratio of 1:1 and incubated at 37°C for 4h. Solution was mixed gently and centrifuged. The pellet obtained by centrifugation was washed thrice with methanol and dissolved in 1 ml each of 2 M KOH and DMSO. Absorbance was recorded at 630 nm. The OD obtained was compared with a standard plot constructed against NBT and ROS generation and values were expressed as µ mole of NBT/mg protein.

Measurement of Lipid Peroxidation
The level of lipid peroxidation (LPO) was determined by measuring the amount of malondialdehyde (MDA), the product of lipid peroxidation, following the method reported earlier [27]. Briefly, 250 µL of the mitochondrial extract was mixed with 250 µL of 0.2 M Tris-Maleate buffer (pH 5.9) and mixture was incubated on water bath at 37°C for 30 min. 750 µL of thiobarbituric acid (TBA) was added in the mixture, was incubated in boiling water bath for 10 min using tight condensers. After the mixture was cooled down, 2 mL of 1 N NaOH were added and allowed to stand for 10 min. The absorbance at 548 nm was recorded and levels of lipid peroxidation were expressed as nmol MDA/mg protein.

SOD Activity Assay
SOD activity was estimated by the method of Kakar et al. [28] Reaction 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 mitochondrial 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.

GPx Activity Assay
Glutathione peroxidase following the method reported [27]. In 1 mL assay buffer (100 mM Tris-HCl (pH 7.2), 3 mM EDTA, 1 mM sodium azide, 0.25 mM H2O2, 0.5 mM NADPH, 0.17 mM GSH, and one unit of glutathione reductase) freshly prepared mitochondrial 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.

GR Activity Assay
GR activity was measured according to the method of Manna et al. [29]. About 100 µl of mitochondrial extract was mixed with 2 mL of 0.2 M KH_2PO_4 buffer containing 1 mM EDTA (pH 7.5), 1 mL of 0.3 mM DTNB, 500 µL water, 200 µL of 2 mM NADPH in water and 200 µL of 20 mM GSSG solution. OD was measured spectrophotometrically at 412 nm for 3 min at 24°C. The enzyme activity was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol NADPH/min.

GSH and GSSG Levels

Method of was used to determine the total glutathione (GSH and GSSG) [30]. Briefly For total glutathione, 200 µL of mitochondrial extract were precipitated with 100 µL of 5% SSA (Sulfosalic acid) and after centrifugation supernatant was neutralized. 50 µL of supernatant was incubated with 100 µL of reagent (0.30 mM NADPH, 0.22 mM DTNB, 1mM EDTA, 1.6 units/ml GR prepared in 100 mM phosphate buffer , pH 7.4) absorbance was recorded at 412 nm for 10 min. To determine the GSSG content separately neutralized solution 100 µL was masked with 2 µL of vinyl pyridine by incubation with 100 µL of the reagent at room temperature for 60 min and absorbance was recorded at 412 nm. Concentration of GSH was determined by subtracting the amount of GSSG from total GSH content.

Caspase-3 Activity

Freshly excised liver was processed as previously described and centrifuged at 10,000 x g for 15 min. A 50 ml appropriately diluted aliquot of supernatant was was assayed using commercial kit "Caspase 3 Assay Kit (Colorimetric) Abcam Canada.

Statistical Analysis

All data were expressed as mean ± SD Statistical analyses were conducted by unpaired Student's *t*-test and. Value of $p<0.05$ were considered as significant. No mortality and alteration in body wt. was observed after the treatment. All the results are based on biochemical studies on mitochondrial extract except Caspase 3 activity.

RESULTS

Effect of Fisetin on the Oxidative Stress and Lipid Peroxidation

ROS level:

Treatment of mice with CP (6 mg/Kg b.w.) resulted significant ($p<0.01$) enhanced in ROS level in mitochondria when compared with control. CP treated mice supplemented with fisetin showed a significant ($p<0.01$) depletion in the ROS level. ROS level in fisetin treated mice showed no significant change in ROS level (Figure 1a).

Lipid peroxidation (MDA level):

Cisplatin treatment significantly ($p<0.001$) enhanced the LPO level in liver mitochondria when compared with control. Cisplatin treated mice supplemented with fisetin showed significant ($p<0.001$) depletion of LPO level. We did not find any change in LPO level in fisetin alone group (Figure 1b).

Effect of Fisetin on Mn-SOD Activity

CP administration significantly ($p<0.01$) increased the Mn-SOD activity in liver mitochondria when compared with control group. CP treated animal supplemented with fisetin showed significant ($p<0.01$) decrease in SOD activity when compared with CP alone group. No significant change was observed in fisetin alone group when compared with control (Figure 2).

Effect of fisetin on Mitochondrial GSH and GSSG

GSH level:

CP treated mice showed a significant ($p<0.001$) decrease in mGSH content in liver mitochondria when compared to control group. CP treated mice supplemented with fisetin showed significant ($p<0.001$) enhanced level of GSH when compared with CP alone group. No significant change in GSH level was observed in the fisetin alone group (Figure 3a).

GSSG level:

CP treated mice showed a significant ($p<0.01$) increased in mGSSG content in liver mitochondria when compared to control group. CP treated mice supplemented with fisetin showed significant ($p<0.01$) declined level of GSH when compared with CP alone group. No significant change in GSSG level was observed in the fisetin alone group (Figure 3b).

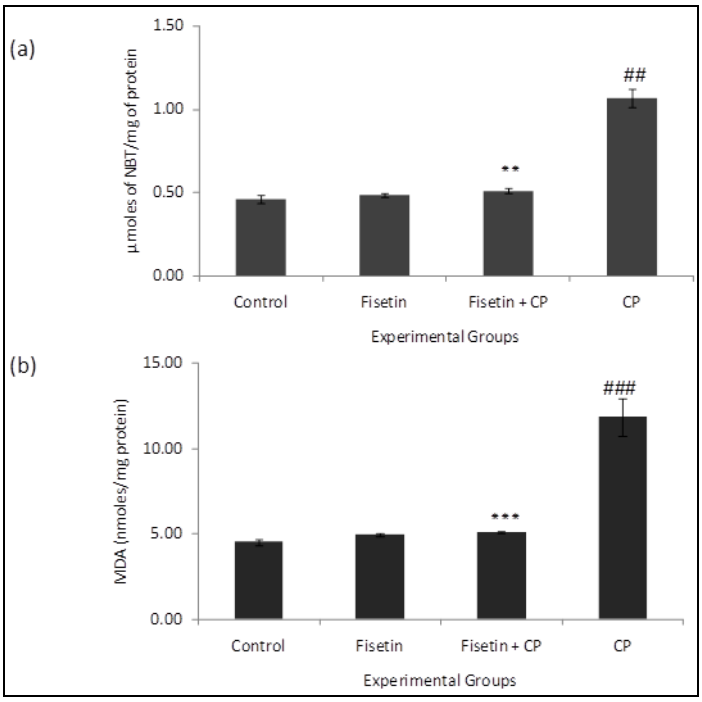

Figure 1: Effect of fisetin on a. ROS and b. LPO in mitochondria. a. Values were expressed as µmole of NBT reduced/mg protein b. Values were expressed as µmole of MDA/mg protein. Each value represents as mean ± SD(n=6). Significant difference were indicated by ##p<0.01, ###p<0.001 (CP vs. Control) and **p<0.01 and p<0.001 (Fisetin +CP vs. CP)

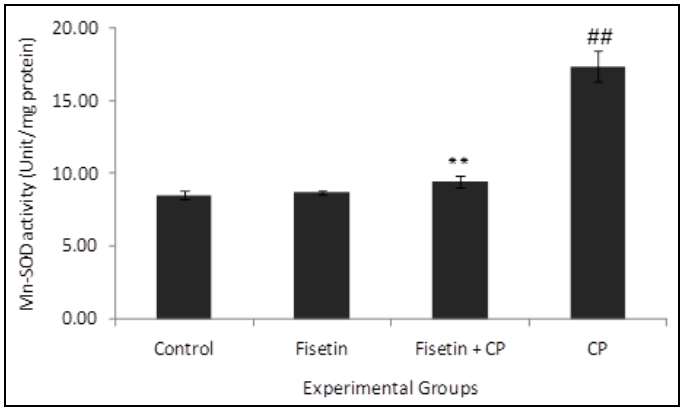

Figure 2: Effect of fisetin on SOD activity in liver mitochondria.Values were expressed as Unit/mg protein Each value represents as mean ± SD(n=6). Significant difference were indicated by ##p<0.01 (CP vs. Control) and **p<0.01 (Fisetin+CP vs. CP)

Effect of Fisetin on GSH Metabolizing Enzymes

Gpx activity:

CP administration significantly ($p<0.001$) increased the GPx activity in liver mitochondria when compared with control group. CP treated animal supplemented with fisetin showed significant ($p<0.001$) decrease in GPx activity when compared with CP alone group. No significant change in GPX activity was observed in fisetin alone group when compared with control (Figure 4a).

GR activity:

CP administration significantly ($p<0.01$) increased the GR activity in liver mitochondria when compared with control group. CP treated animal supplemented with fisetin showed significant ($p<0.01$) decrease in GR activity when compared with CP alone group. No significant change in GR activity was observed in Fisetin alone group when compared with control (Figure 4b).

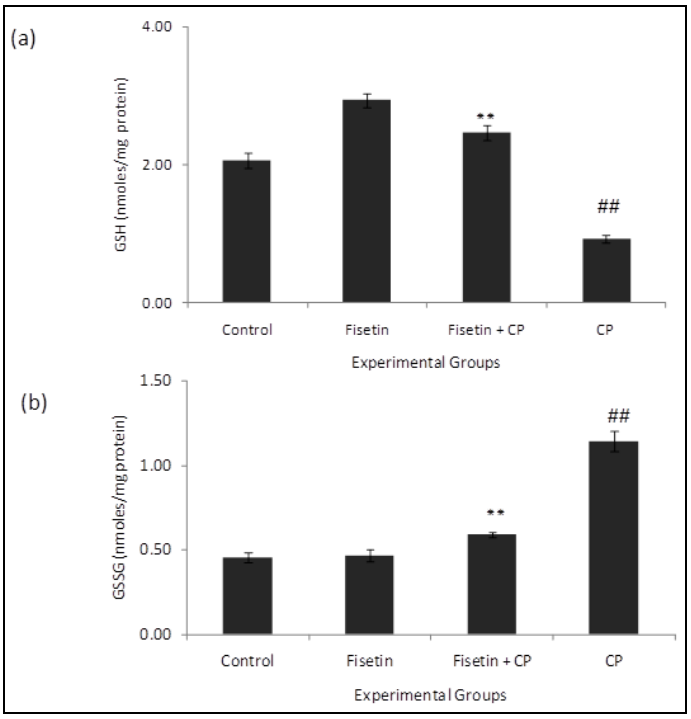

Figure 3**: Effect of fisetin on a. GSHand b. GSSG in mitochondria. a. Values were expressed as Values were expressed as nmole of/mg protein. Each value represents as mean ± SD(n=**6). **Significant difference were indicated by ##p<**0.01**, ###p<**0.001 **(CP vs. Control) and **p<**0.01 **and ***p<**0.001 **(Fisetin+CP vs. CP)**

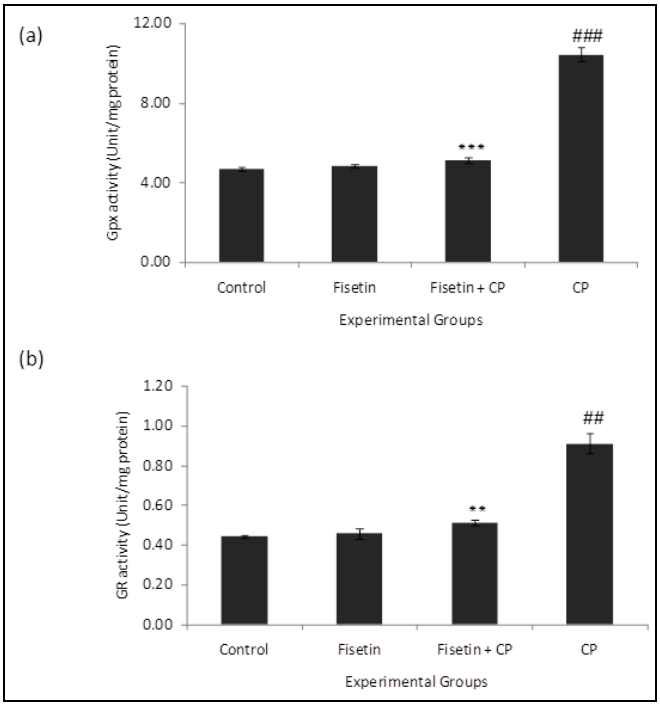

Figure 4**: Effect of fisetin on GSH metabolizing enzymes a. Gpx and b. GR in liver mitochondria.Values were expressed as were expressed as activity of enzymes Unit/mg protein. Each value represents as mean ± SD(n=**6). **Significant difference were indicated by ##p<**0.01**, ###p<**0.001 **(CP vs. Control) and **p<**0.01 **and ***p<**0.001 **(Fisetin+CP vs. CP)**

Effect of Fisetin on the Caspase 3 Activity Induced by Cisplatin

CP administration significantly ($p<0.001$) increased the caspase 3 activity in cytosolic fraction when compared with control group. CP treated animal supplemented with fisetin showed significant ($p<0.01$) decrease in caspase 3 activity when compared with CP alone group. No significant change was observed in Fisetin alone group when compared with control (Figure 5).

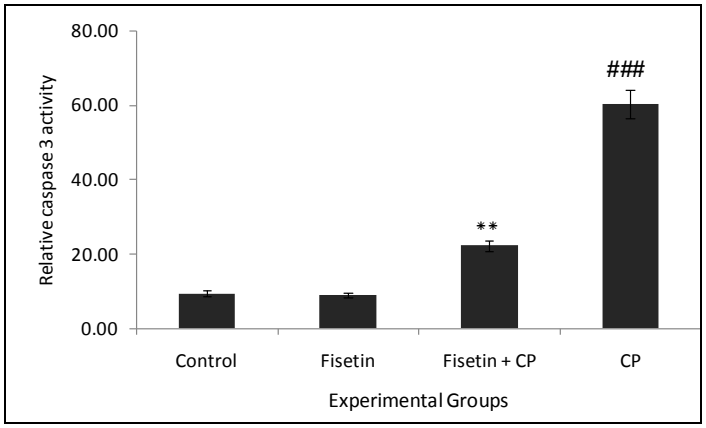

Figure 5: Effect of fisetin on Caspase 3 activity in cytosol liver. Values were expressed as Relative activity .Each value represents as mean ± SD(n=6). Significant difference were indicated by ###p<0.01 (CP vs. Control) and **p<0.01 (Fisetin+CP vs. CP)

DISCUSSION

Mitochodrial oxidative stress is due to accumulation of ROS. CP induced ROS accumulation is also reported in mitochondria [1]. ROS are highly reactive molecule if its level is not restricted it induces mitochondrial dysfunction [2]. Fisetin supplementation also depleted CP induced ROS (Figure 1a). Fisetin, a dietary flavonol, has four hydroxyl group thus proposed to act as important quencher of ROS [22]. It has been reported that fisetin could able to quench H_2O_2 and radiation induced ROS accumulation *in virto* [31,32]. ROS is also associated with inactivation of complex IV of electron transport chain complex thereby mitochondrial dysfunction. Thus, our study also suggests the antioxidant activity of fisetin (Figure 1a). Lipids are most preferred molecule which undergoes lipid peroxidation (LPO) by ROS. LPO has been hypothesized to be a mechanism of cellular damage [33]. *In silico* studies suggest that fisetin has special protective effect against lipid peroxidation (LPO) [34]. Increased level of LPO in mitochondrial matrix in CP group is due to increases ROS level (Figure 1b). Cardiolipn is an anionic phospholipid, which is specifically found in mitochondria. Cardiolipin plays important role in cell death regulation because it is associated with cytochrome c [32]. In addition truncated lipids are prerequisite to tumor necrosis factor induced apoptosis [21]. Increased level of LPO also proposed the peroxidation of cardiolipin because ROS are not specifically reactive to macromolecules. LPO predispose mitochondrial membrane to apoptosis by cytochrome c leakage in cytosol and caspase activation [19] Fisetin has been reported to protect LPO against ROS in RBC [20]. Our result also demonstrates the LPO protective activity of fisetin and declined caspase 3 activities also support the mechanism (Figures 1b and 5). Thus, fisetin could act compensatory mechanism to protect mitochondrial LPO against ROS.

ROS is modulated by enzymatic and nonenzymatic components of cell. Mn-SOD is mitochondrial enzyme which catalyses the ROS to hydrogen peroxide. CP administration increased the activity of Mn-SOD to quench the increased level of ROS (Figure 3). Fisetin treatment declined the activity of Mn-SOD might be due to decreased level of ROS in the mitochondria (Figure 3). These results further suggest the ROS quenching activity of fisetin [32].

mGSH level is an important indicator of oxidative stress conditions in mitochondria. Depletion of mGSH has been shown to be associated with genobiotic toxicity [35]. GSH assist to quench ROS [2]. CP induced declined mGSH level in mitochondria because mGSH pool is utilized to detoxify increased ROS level and sufficient GSH pool is not available in cytosol. It has reported that fisetin increases the GSH level in cytosol [23] although we did not find any change in mGSH level (Figure 4a). This might be due to saturation of GSH transport to mitochondria under normal conditions. In addition, mGSH depletion in hepatocyte exposed to acetoaminophen had shown mGSH level preceded that of cytosol [36,37]. It has been reported that fisetin modulated the level of total GSH in the cell and activity of antioxidant enzymes in liver [23,38]. Fisetin enhanced the level of mGSH during CP toxicity and this is very likely because fisetin increases the GSH level in cytosol (Figure 4a). In addition, threshold GSH level is needs

to be maintained in mitochondrial matrix [39] because mitochondrial proteins are acclimatized to live in reducing pool of GSH [2]. Mitochondrial protein undergoes spontaneous glutathiolation to maintain normal physiological function of mitochondria [13]. CP administration shifts the reducing GSH to oxidizing GSSG environment of mitochondrial matrix (Figure 4b) [1]. GSH/GSSG ratio clearly indicate that CP treatment shift the GSH reducing pool of mitochondrial matrix to GSSG oxidizing (Figures 4a and 4b). This leads to affect the specific glutathiolation of critical proteins thereby mitochondrial dysfunction [13]. Fisetin could able to maintain normal GSH reducing pool and hence normal mitochondrial function.

Glutathione peroxidase 4 is mitochondrial isoform which directly quench hydrogen peroxide [6,40,41]. It has been reported that it is associated with inner mitochondrial membrane thus critically important to protect lipid against ROS induced peroxidation [40]. Increased activity of GPx in mitochondria is protective mechanism against peroxidation. This might be also a compensatory mechanism against depletion of mGSH. In Fisetin treated group declined activity of Gpx because on one hand fisetin reduced the level of ROS & LPO on other hand it increases mGSH level (Figure 5). NADPH dependent enzyme glutathione reductase (GR) recycles GSSG back to GSH. Similar behavior of GR was observed in the experiment because it is downstream enzyme cascade to GPx (Figure 5). Therefore, down regulation of Activity of Gpx and GR again support the antioxidant potential of fisetin.

Summary of Effect of Fisetin on Antioxidant Status of Mitochondria

Fisetin modulated the mGSH level and restores the antioxidant enzyme activity in mitochondria that gives protection against CP induced oxidative stress. Consequence of this is protection of LPO in mitochondria and reduced caspase3 activity in cytosol.

CONCLUSION

Natural product could play critical role in treatment of drug induced toxicity [42]. 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 [22] but not in mitochondria. Fisetin restore mGSH level thereby given protection against CP induced mitochondrial oxidative stress. Effect of fisetin on mGSH need to be explored further because our study indicates the fisetin might affect the transport of GSH [20]. Major limitation of this study is that we cannot able to separately present the antioxidant effect of fisetin and its effect on GSH transport. Our study gives an indication to look for targeting mGSH as a new therapeutic direction.

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