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Imbalance Between Peroxisomal ROS Generating Enzymes and ROS Scavenging Enzymes May Contribute to Cyclophosphamide Induced Renal Damage in Rats

KEYWORDS: Cyclophosphamide, kidney, peroxisomes, ROS generating enzymes

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ABSTRACT *The mechanism of renal toxicity of cyclophosphamide (CP), a commonly used anticancer drug is not clear. In a recent study we have demonstrated increase in size and number of peroxisomes in the kidneys of rats after treatment with cyclophosphamide. Our aim is to examine whether there is any alterations in the expressions of peroxisomal ROS metabolising enzymes in the kidneys of rats upon CP treatment. Rats received single i.p. injection of 150 mg/kg body wt. and killed 24 hrs.later. The protein expression and activities of ROS generating enzymes (xanthine oxidase, fatty acid oxidase, D amino acid oxidase, polyamine oxidase, and urate oxidase) and activities ROS scavenging enzymes (catalase, glutathione peroxidase and glutathione transferase) were determined in the kidney peroxisomal fractions by western blot and spectrophotometric methods respectively. A significant increase in the protein levels of the ROS generating enzymes (except urate oxidase) was observed in the peroxisomal fractions of CP treated rat kidneys. The activity of catalase was increased, while the activities of other antioxidant enzymes remained unaltered upon CP treatment. It is concluded that peroxisomal dysfunction i.e. imbalance between the ROS generating enzymes and ROS scavenging enzymes may contribute to CP induced renal damage.*

Introduction

Cyclophosphamide (CP) is a drug with a wide spectrum of clinical uses and it has been proved to be effective in the treatment of cancer and non-malignant disease states such as rheumatoid arthritis (**Reiter, 2008; Palumbo and Rajkumar, 2010**). However, this drug may induce acute inflammation of the urinary bladder, renal damage, and liver damage, thereby limiting the therapeutic use of the drug. Studies have shown that CP can be nephrotoxic, both in humans and animal models. CP can result in glomerular dysfunction and tubular dysfunction. **(Kopecna, 2001; Pe´rez-Sua´rez et al, 2009)**. The mechanism of renal toxicity of CP is not clear although our studies and others have shown that oxidative stress and nitrosative stress play a role (**Stankiewicz and Skrzydlewska, 2003; Abraham and Sugumar, 2008; Abraham and Rabi, 2009, 2011; Merwid-Ląd et al, 2012).**

Initially, it was assumed that the main function of peroxisomes was the decomposition of H_2O_2, a reactive oxygen species (ROS) generated by different peroxisomal oxidases via catalase, the classical peroxisomal marker enzyme. However, it is now clear that peroxisomes are involved in generation of ROS. The main metabolic processes contributing to the generation of H_2O_2 in peroxisomes are the fatty acyl CoA oxidase(ACOX), xanthine oxidase (XO), urate oxidase(UO), polyamine oxidase (PAOX) and D amino acid oxidase (DAAO) **(Antonenkov et al, 2010)**. In our earlier study we have demonstrated peroxisome proliferation in the kidneys of CP treated rats **(Abraham and Isaac, 2011)**. We hypothesised that peroxisomal dysfunction may contribute to CP induced renal damage. Therefore in the present study we examined the protein levels and activities of some important ROS generating peroxisomal enzymes along with the activities of peroxisomal antioxidant enzymes namely catalase, glutathione peroxidase (GPO) and glutathione S transferase (GST).

The results of the present study show that CP administration results in peroxisomal dysfunction in the kidneys of rats as evidenced by altered protein and enzyme activities of ROS generating as well as ROS scavenging enzymes. It is suggested that the imbalance in ROS generating and ROS detoxifying peroxisomal enzymes may contribute to CP induced renal damage in rats.

Materials and methods

Chemicals

Cyclophosphamide (endoxan) was purchased from Zydus Biogen, Ahmedabad, India. All other chemicals were of analytical grade and were purchased from Sigma Chemical Co., St. Louis, MO, USA and Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai, India.

Animals and treatments

Adult male Wistar rats (200–250 g) were used for the experiments. The study was approved by the animal ethics Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India and the institutional research board. The guidelines were followed. The rats were maintained in a controlled environment at 20˚C and 40%–50% humidity, with 12 hrs of light per 24-hr period. Rodent diet and tap water were consumed by rats *ad libitum*.

Experimental design

The rats were randomly divided into 2 groups and were treated as follows:

Group I (control): The rats in this group (n = 6) received saline i.p.

Group II: The rats (n =8) in this group received 150 mg/kg body weight CP in saline i.p.

The rats were killed 24 hrs after the administration of CP. The kidneys were removed, blotted dry, and processed for electron microscopic studies and biochemical studies.

Dosage and route of administration of CP were deter-

Volume : 6 | Issue : 11 | November 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

mined from that described in literature **(Ahluwalia et al .1994)**.

Scanning electron microscopy of kidney epithelium
The kidneys were fixed in 3% glutaraldehyde and washed in 0.1 M sodium cacodylate buffer pH 7.4, post-fixed by 1% osmium tetraoxide and washed in buffer, and dehydrated in increasing concentrations of alcohol. The tissues were processed and examined with transmission electron microscope (Philips 201C by Netherland) and photographed. Both the glomeruli and tubules were examined.

Preparation of a peroxisome-enriched fraction from tissues.
A peroxisome-enriched fraction was prepared from rat kidneys as described by **Yu et al, 1998**. Briefly, using a Potter-Elvejhem homogenizer, frozen tissues were homogenized in five volumes of ice-cold homogenization buffer containing 5 volumes of 67 mM phosphate buffer, pH 7, containing 1 %(v/v) Triton –X 100. The homogenates were subjected to differential centrifugation by using a Beckman centrifuge. The peroxisome-enriched fractions from the tissues were resuspended in 0.5 ml of the same ice-cold homogenization buffer per gram of tissue, and samples were used for western blotting.

Western blotting
Western blotting was performed analyzing samples (5-20 µg protein) on 7.5 -15 % polyacrylamide denaturing gels in accordance with Laemmli. Membranes were incubated overnight at 4° C with the following primary antibodies appropriately diluted in blocking solution: 1:1000 anti-XO; 1:2000 anti PAOX; 1:1000 anti-DAAO ; 1:1000 anti-UO ; 1:2000 anti-ACOX and 1: 2000 anti-β actin. This was followed by incubation with 1:2000 HRP-conjugated anti rabbit IgG secondary antibody in blocking solution, for 1 hr. at 4˚ C. After rinsing, immunoreactive bands were visualized by means of enhanced chemiluminescence according to the manufacturer's instructions. The relative densities of the immunoreactive bands were determined and normalized with respect to β actin, using a semiquantitative densitometric analysis.

Enzymatic activity assays
The kidney tissues were homogenized in 5 volumes of 67 mM phosphate buffer, pH 7, containing 1 % (v/v) Triton –X 100, and centrifuged at 13000 rpm for 30 minutes at 4˚ C to obtain the supernatant containing peroxisomes. The resulting extracts were used for spectrophotometrical enzymatic activity measurements of XO **(Terada et al. 1992)**, UO **(Arima and Nose, 1968),** DAAO **(Nagata et al, 1988)**, catalase **(Aebi, 1984)**, glutathione peroxidase (GPO) **(Nakamura and Hosada, 1974),** glutathione S transferase (GST) **(Awasthi et al, 1980)** and protein measurements **(Lowry et al, 1951).**

2.8. Statistical analysis: The data represent mean value ± S.D. Means. Student's *t* test with Bonferroni correction was used to compare individual means. A P value < 0.05 was considered statistically significant.

Results
Ultrastructural changes in the kidney
The glomerulus of the control rats appeared normal (Fig.1a). The proximal convoluted tubule showed normal brush border. The cells lining the tubule had a number of mitochondria (normal feature) and lysosomes (Fig. 1b). Treatment with CP resulted in remarkable damage to cells of the glomerulus and the tubules. In the glomerulus, there was vacuolization of cytoplasm of the mesangial cells (Fig.1c). In the tubules, many peroxisomes were seen and they were enlarged. Polymorphic mitochondria were increased in number. There were very few lysosomes (Fig.1d).

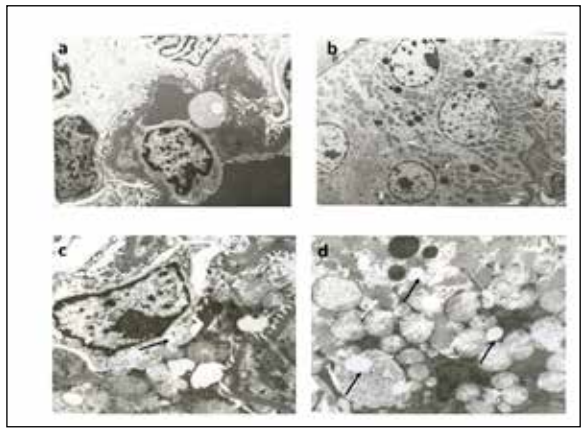

Fig.1. Electron microscopic appearance of glomerulus and tubules of control rats and cyclophosphamide treated rats. (a) Glomerulus of control rat showing normal structure, X 1500. (b) Proximal tubule of control rat kidney showing numerous mitochondria and lysosomes, X 1500. (c) Glomerulus of CP treated rat showing vacuolization of cytoplasm of the mesangial cells (black arrow) (d) Proximal tubule of CP treated rat kidney showing increase in the number and size of peroxisomes (black arrow), polymorphic mitochondria, round vacuoles filled with opaque substance (white arrow), X 10,000.

Effect of CP treatment on the protein levels of peroxisomal ROS generating enzymes in the kidney

The protein levels of all the peroxisomal ROS generating enzymes assayed (except UO) were significantly increased in the kidneys of CP treated rats as compared with control. With respect to xanthine oxidase and fatty acyl CoA oxidase (ACOX), a 5 fold increase (fig.2) and 1.4 fold increase was observed respectively in the CP treated rats as compared with control (fig.3).

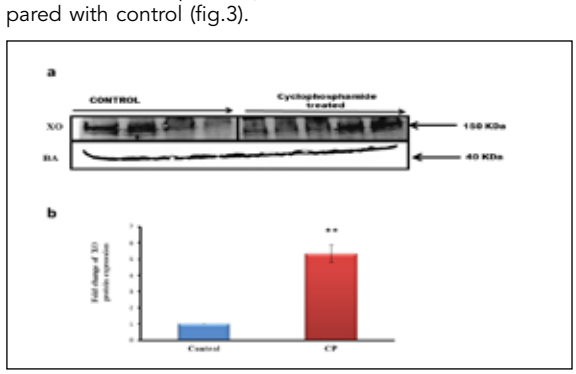

Fig.2. Representative western blots of renal xanthine oxidase (XO) protein levels in control and CP-treated rats (n=5 in each group), as assessed by using a 10% polyacrylamide gel, loading 100µg protein per lane. β-actin (BA) was used as the loading control. b. Relative concentration of xanthine oxidase protein in renal homogenates as analysed by densitometric quantification of bands of western blots in control and CP treated rats. Data represents mean ± SD, obtained by image analysis of western

Volume : 6 | Issue : 11 | November 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

blots with the concentration of the controls set at one. ** p<0.01 when compared with control values

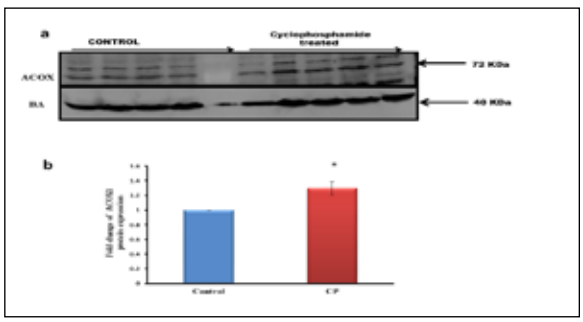

Fig.3.Representative western blots of renal ACOX protein levels in control and CP-treated rats (n=5 in each group), as assessed by using a 10% polyacrylamide gel, loading 100µg protein per lane. β-actin (BA) was used as the loading control. b. Relative concentration of ACOX protein in renal homogenates as analysed by densitometric quantification of bands of western blots in control and CP treated rats. Data represents mean ± SD, obtained by image analysis of western blots with the concentration of the controls set at one. * p<0.05 when compared with control values

With respect to DAAO and PAOX a 1.3 fold increase (fig.4) and 1.75 fold increase (Fig.5) was observed respectively in the CP treated rats. However surprisingly, a 5 fold decrease in uric acid oxidase protein levels was observed in the CP treated rat kidneys (Fig.6).

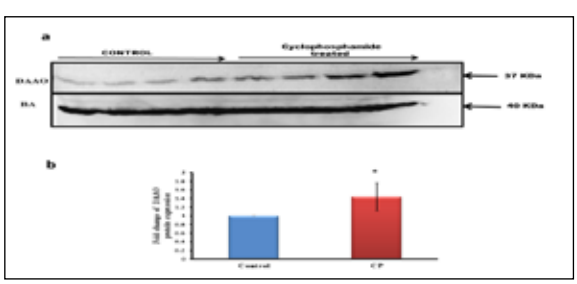

Fig.4. Representative western blots of renal DAAO protein levels in control and CP-treated rats (n=5 in each group), as assessed by using a 10% polyacrylamide gel, loading 100µg protein per lane. β-actin (BA) was used as the loading control. b. Relative concentration of D amino acid oxidase protein in renal homogenates as analysed by densitometric quantification of bands of western blots in control and CP treated rats. Data represents mean ± SD, obtained by image analysis of western blots with the concentration of the controls set at one. * p<0.05 when compared with control values

Fig.5. Representative western blots of renal PAOX protein levels in control and CP-treated rats (n=5 in each group), as assessed by using a 10% polyacrylamide gel, loading 100µg protein per lane. β-actin (BA) was used as the loading control. b. Relative concentration of PAOX protein in renal homogenates as analysed by densitometric quantification of bands of western blots in control and CP treated rats. Data represents mean ± SD, obtained by image analysis of western blots with the concentration of the controls set at one. * p<0.05 when compared with control values

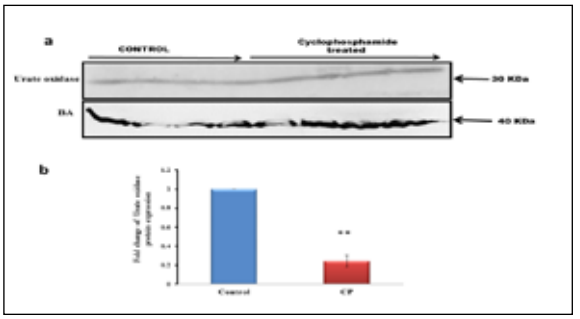

Fig.6. Representative western blots of renal UO protein levels in control and CP-treated rats (n=5 in each group), as assessed by using a 10% polyacrylamide gel, loading 100µg protein per lane. β-actin (BA) was used as the loading control. b. Relative concentration of UO protein in renal homogenates as analysed by densitometric quantification of bands of western blots in control and CP treated rats. Data represents mean ± SD, obtained by image analysis of western blots with the concentration of the controls set at one. ** p<0.01 when compared with control values

3.4. Effect of CP treatment on the enzyme activities of peroxisomal ROS generating enzymes in the kidney

To our surprise, increase in protein levels of ROS generating enzymes did not translate into increase in the activities of these enzymes (fig.7). The activities of XO and DAA oxidase were not significantly altered in the CP treated rats as compared with control (fig.7 a &b). However, the activity of uric acid oxidase was decreased significantly in the CP treated rats as compared with control (Fig.7 c).

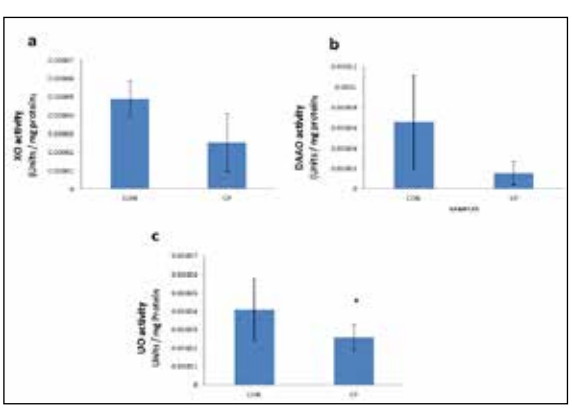

Fig 7. a. Xanthine oxidase activity in the peroxisomal fractions of the kidneys of control rats and CP treated rats. Data represent mean ± SD, n= 6 in each group. b. D amino acid oxidase activity in the peroxisomal fractions of the kidneys of control rats and CP treated rats. Data represent mean ± SD, n= 6 in each group. c. Urate oxidase activity in the peroxisomal fractions of the kidneys of control rats and CP treated rats. Data represent mean ± SD, n= 6 in each group. * p<0.05 when compared with control values.

3.5. Effect of CP treatment on the enzyme activities of peroxisomal ROS scavenging enzymes

We assayed the activities of catalase, GPO and GST, the peroxisomal enzymes involved tin the detoxification of ROS. A significant increase in the activity of catalase was observed in the kidneys of CP treated rats (fig. 8a). We could not find any significant difference in the activities of

Volume : 6 | Issue : 11 | November 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

GPO and GST between the CP treated rats and control rats; however, we found increasing trend in the activities of these enzymes in CP treated rats as compared with control (figs. 8b &c).

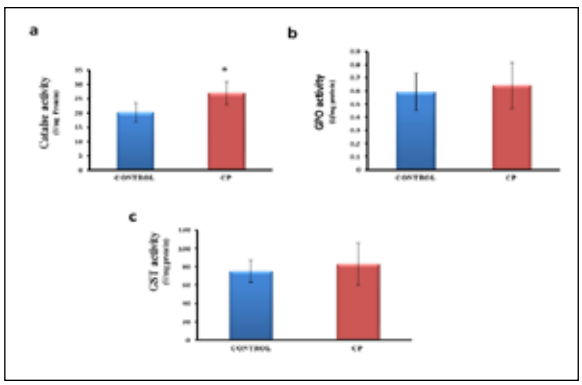

Fig.8. a. Catalase activity in the peroxisomal fractions of the kidneys of control rats and CP treated rats. Data represent mean ± SD, n= 6 in each group. * p<0.05 when compared with control values.b. GPO activity in the peroxisomal fractions of the kidneys of control rats and CP treated rats. Data represent mean ± SD, n= 6 in each group. c. GST activity in the peroxisomal fractions of the kidneys of control rats and CP treated rats. Data represent mean ± SD, n= 6 in each group.

Discussion

Cyclophosphamide (CP) is a drug with a wide spectrum of clinical uses and it has been proved to be effective in the treatment of cancer and non-malignant disease states such as rheumatoid arthritis **(Reiter, 2008 ; Palumbo A and Rajkumar, 2010).** Nephrotoxicity is a major dose-limiting side effect of CP **(Pe´rez-Sua´rez et al, 2009)**. The mechanism of renal toxicity of CP is not clear, although our studies and others have shown that oxidative stress and nitrosative stress play a role **(Stankiewicz and Skrzydlewska, 2003; Abraham and Sugumar, 2008; Abraham and Suganthy, 2009,2011; Merwid-Ląd et al, 2012**).

Peroxisome proliferation is usually characterized by an increase in the number and size of peroxisomes, and an induction of peroxisomal enzymes, especially those involved in fatty acid β-oxidation **(Lazarow and De Duve, 1976)**. Accordingly in our earlier study we were able to demonstrate increase in number and size of peroxisomes in the kidneys of CP treated rats **(Abraham and Isaac, 2011)**.

A common product of the catabolic reaction is the generation of H_2O_2. Peroxisomes generate significant amounts of hydrogen peroxide (about 35% of all H_2O_2 produced in rat liver) through the action of several peroxisomal oxidases (e. g., their acyl-CoA oxidases), that can be converted to more aggressive ROS **(Boveris et al, 1972)**. However, peroxisomes also contain multiple antioxidant enzymes (e. g., catalase, Cu Zn-SOD, glutathione peroxidase, epoxide hydrolase, peroxiredoxin I, MnSOD) that contribute to the regulation of intracellular ROS levels and thus oxidative stress. The massive peroxisome proliferation induced by a variety of peroxisome proliferators and the subsequent tumor formation in rodents is thought to be due to imbalance in the formation and scavenging of ROS generated by peroxisomes **(Reddy and Rao, 1989**).

In the present study, we were able to demonstrate increase in amount of the peroxisomal ROS generating enzymes namely fatty acid oxidase, xanthine oxidase, polyamine oxidase and D amino acid oxidase.

The β oxidation of fatty acids is the most important metabolic process in the peroxisomes contributing to the formation of H_2O_2 **(Hiltunen and Poirier, 2006)**. In the present study, protein level of fatty acid oxidase was significantly increased in the kidneys of CP treated rats suggesting that it may be one of the sources of ROS. Peroxisomal β-oxidation and imbalance in the cellular redox state is associated with ROS overproduction during various pathophysiological conditions such as inflammation, ischemia-reperfusion, diabetes and hepatic allograft rejection and acute kidney injury **(Gulati et al, 1992; Asayama et al, 1999; Vasko et al, 2013)**.

XO is a complex molybdoflavoprotein which is rate limiting enzyme in purine catabolism wherein it utilises hypoxanthine and xanthine as substrates. The by-products of this reaction are O_2 and H_2O_2 and superoxide **(Engerson et al, 1987)**. In addition, this enzyme catalyses the reduction of nitrates and nitrites to nitric oxide, thus acting as source of both nitric oxide (NO) and its toxic product peroxynitrite (PON) **(Harrison, 2002)**. It is worthwhile to mention here that in the present study we found 5 fold increase in XO protein in the CP treated rat kidneys. It is also important to mention here that in our earlier studies we have demonstrated NO and PON overproduction, increased nitrosative stress in the kidneys of CP treated rats **(Abraham and Sugumar, 2008; Abraham and Rabi, 2009, 2011)**. It is suggested that increase in XO may contribute to increased oxido-nitrative stress seen in CP treated rat kidneys.

D amino acid oxidase was one of the first enzymes detected in mammalian peroxisomes **(DeDuve and Baudhuin, 1966**). D Amino acid oxidase in mammal kidney regulates the renal reactive oxygen species (ROS) levels directly and plays a leading role in the development of ROS-mediated renal pathologic damages **(Zhang et al , 2012)**. In the present study the protein level of D amino acid oxidase was increased in the kidneys of CP treated rats as compared with control. The increased level of this enzyme may contribute to ROS production.

The polyamines- putrescine, spermine and spermidine are essential for growth, function and maintenance of mammalian cell **(Moinard et al, 2005)**. They are oxidised by PAOX and H_2O_2 is one of the by products **(Wu et al, 2003)**. In the present study increased polyamine oxidase was observed in the kidneys of CP treated rats. The increased polyamine oxidase may contribute to ROS production in the kidneys of CP treated rats.

Urate oxidase is a cuproprotein and catalyses the oxidation of uric acid to allantoin with the formation of H_2O_2. The natural crystals of urate oxidase are detected only in liver and kidney peroxisomes **(Usuda et al, 1988**). In the present study, unlike the protein levels of other ROS generating peroxisomal oxidases, we found significant decrease in uric acid oxidase protein levels in the kidneys of CP treated rats as compared with control. Uric acid is a powerful antioxidant that scavenges singlet oxygen, peroxynitrite and free radicals (Ames et al, 1981; Hooper et al, 2000). It is suggested that loss of urate oxidase may be beneficial as it allows the accumulation of uric acid **(Ames et al, 1981)**. Therefore the decreased uric acid oxidase protein in the kidneys of CP treated rats may be a defence mechanism to combat increased ROS and RNS produced as a result of CP treatment.

Volume : 6 | Issue : 11 | November 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

We next assayed the activities of some oxidases i.e. XO, DAAO and UO in the peroxisomal fractions of rat kidney homogenates. The activities of XO and DAAO were not altered in the kidneys of CP treated rats. It is not clear why the activities of the oxidases were not altered significantly although their protein levels were increased in the kidneys of CP treated rats. A study showed that the operation of renal ischemia resulted in a 45 % decrease in the DAAO-induced consumption of substrate, indicating a sharp decrease in renal DAAO activity following this acute renal injury (**Zhang et al , 2012**). In the present study a significant decrease in the activity of urate oxidase was observed. The decrease in urate oxidase activity may result in the accumulation of uric acid, an important antioxidant in the kidney. This may be an adaptive mechanism to combat ROS and RNS that are generated upon CP administration.

We next investigated whether there is alteration in the activities the peroxisomal antioxidant enzymes catalase, GPO and GST. The activity of catalase was increased in CP treated rat kidneys, while the others remained unaltered. Since properly functioning catalase is indispensable for neutralization of H_2O_2 generated, the increase in its activity may an adaptive mechanism.

Conclusion

The results of the present study show that CP treatment results in peroxisomal proliferation and peroxisomal dysfunction in the kidneys of rats. Because of the central role of peroxisomes in ROS production and the catabolism of inflammatory lipid mediators such as harmful metabolites of arachidonic acid or leukotriene B4 (**Ferdinandusse et al, 2002; Hall et al, 2010**), the impairment of peroxisomal function upon CP treatment can contribute significantly to the prolongation and intensification of inflammatory process. The mechanism by which CP causes peroxisomal proliferation and dysfunction is not clear. Further biochemical and molecular studies may lead to a better understanding of the mechanisms and implications of peroxisome proliferation and dysfunction in CP induced renal damage.

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Volume : 6 | Issue : 11 | November 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

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