International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **2(12),** 42-49, December **(2013)** *Int. Res. J. Biological Sci.*

Hepatoprotective Role of Curcumin against Acetaminophen induced toxicity in rats

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Available online at: www.isca.in, www.isca.me
Received 30th July 2013, revised 10th August 2013, accepted 8th October 2013

Abstract

Acetaminophen (APAP) induced hepatotoxicity causes severe hepatic damages for which no specific treatment is available. Consequently, the present study is aimed at evaluating the antioxidant effects of curcumin (CMN) on APAP-exposed hepatic damages. Adult male Wistar Albino rats were treated orally with different concentration (0.0 mM -50 mM) of APAP intraperitoneally to induce hepatotoxicity. Twenty-four hours post administration of acetaminophen; rats were sacrificed to measure hepatocytes viability, level of malondialdehyde (MDA), calcium ATPas activity and antioxidant enzyme activity. Results suggest that with an increase in APAP concentration there was decrease in hepatocytes viability, calcium ATPase activity but marked elevation of malondialdehyde. CMN is a well known nutraceutical with potent antioxidant properties. CMN markedly increased hepatocytes viability, calcium ATPase activity and reduced the elevated malondialdehyde levels. The results indicate the therapeutic importance of CMN in overcoming acetaminophen induced hepatotoxicity. It is therefore necessary to explore the role of CMN in treatment of hepatic damages.

Keywords: Oxidative stress, acetaminophen, cell viability, lipid peroxidation, antioxidants, curcumin.

Introduction

Acetaminophen (APAP) popularly known as paracetamol, is indiscriminately and widely used everywhere in the world as a common analgesic and antipyretic drug. It is the most commonly reported toxic ingestion in the United States[1,2]. Nevertheless, it is safe at therapeutic dosages, an overdose of this drug has a high potential for causing hepatotoxicity. This "benign" drug is easily available for suicidal attempts possibly due to easy availability and over the counter purchase. It has largely been observed that suicide attempts or accidental overdose are primarily responsible for most of the cases of APAP poisoning[3]. Moreover, reports indicate that even borderline high doses are hepatotoxic for some infants[4]. In addition, APAP has been associated with acute tubular necrosis, pancreatitis, and myocardial necrosis[5].

The liver and other aerobic organs generate reactive oxygen species (ROS) which is a major cause of oxidative tissue damage. These radicals induce lipid peroxidation (LPO) and damages cell membranes or cause inflammations which have been implicated as important pathological mediators in many clinical disorders such as heart disease, diabetes, gout and cancer[6]. The antioxidant enzymes confer protection by converting active oxygen molecules into non-toxic compounds[7]. Despite this, liver diseases still remains a serious health problem. In allopathic medical practices liver protective drugs are limited. For the management of various liver disorders, herbal drugs plays an important role in enhancing the natural healing processes of the liver. In ethnomedical practice as well as traditional system of medicine in India a number of formulations of medicinal plants are used for liver disorders[8].

In spite of the tremendous advances in modern medicine, there are no effective drug(s) available that stimulates liver function, offer protection to the liver from damage or help to regenerate hepatic cells[9]. Therefore, it is necessary to search for alternative drugs for the treatment of liver diseases to replace currently used drugs of doubtful efficacy and safety. Curcumin (CMN), commonly called diferuloyl methane, is a hydrophobic polyphenol derived from the rhizome (turmeric) of the herb *Curcuma longa* and chemically, it is a bis-a, b-unsaturated b-diketone that exhibits keto-enol tautomerism. It exhibits anticarcinogenic, antimicrobial, antioxidant and anti-inflammatory activities. Besides these it also has hepatoprotective, nephroprotective, hypoglycaemic, antirheumatic activities, suppresses thrombosis and protects against myocardial infarction. Interestingly, CMN has been shown to be extremely safe even at very high doses in various animal models and human studies[10, 11]. Thus, the aim of this study was to investigate the protective effects of CMN on the experimentally APAP - induced hepatotoxicity.

Material and Methods

Animals: Male rats (150–200 g body weight, 6–9 weeks old) were fed ad libitum and had free access to drinking water were maintained under standard conditions in the Department of Biochemistry, Dr. Ram Manohar Lohia Avadh University, Faizabad, U.P., India.

Chemicals: Acetaminophen, CMN and ethylene glycol bis tetra acetic acid (EGTA) and all others chemicals and reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA), BDH Ltd., Merck India Ltd., Ranbaxy India Ltd. and Qualigens India Ltd.. HPLC grade quartz double distilled water was used throughout the study.

Isolation of hepatocytes: Hepatocytes were prepared by a modification of the method of Moldeus et al[12]. Rats and mice were anesthetized with phenobarbital, and the peritoneal cavity was opened and a loose ligature was placed around the portal vein, an oblique incision was then made in the mesenteric part of the vein, and the cannula was immediately inserted. The perfusion was initiated in situ at flow rate of 122 ml / min. The liver was cut free, immersed in buffer at 37°C. Liver were perfused for 5 min. with a modified Hanks buffer (pH 7.4) (136mM NaCl, 5mM KCl, 1mM $MgSO_4$, 0.3mM $NaHCO_3$) containing 0.5mM EGTA, 25mM HEPES (free acid), and 2% BSA. Subsequently liver were perfused with a modified Hanks buffer containing 0.12% collagenase, 4mM $CaCl_2$ and 2% BSA for an additional 10 min. At the end of perfusion, the liver which was swollen and pale was detached from canula and cell were dispersed in a Krebs – Henseleit buffer (118.1mM NaCl, 48mM KCl, 1mM KH_2PO_4, 2.9 mM $CaCl_2$, 23.8 mM $NaHCO_3$, 25mM HEPES) containing 2 mM glutamine, MEM amino acid and MEM vitamins, 1.7 mM glucose, 10% calf serum and 2% BSA (incubation medium). Cells were then filtered through a nylon mesh (150 µm) and allowed to settle after 5 min. the excess medium was aspirated, cells were resuspended in 20 ml of fresh incubation medium and washed twice (200g centrifugation). The hepatocytes were incubated at a concentration of 1 x 10^6 cells per ml in Erlenmeyer flask at 37^0C and gassed with 95% oxygen and 5% CO_2.

Cell Viability Assay: Mitochondrial dehydrogenase activity of isolated hepatocytes in terms of Methyl Thiazol Tetrazolium (MTT) reduction: Cell viability of isolated hepatocytes was checked in terms of MTT reduction. The cell viability of isolated hepatocytes was monitored according to the method described by Mossman et al[13]. MTT stock solution (5mg/ml) was added to each culture being assayed and incubated for 2 hr. At the end of incubation period, the medium was removed and the converted dye was solubilised with acidic isopropanol. Absorbance of converted dye was measured at wavelength 530 nm with background subtraction of 690 nm using spectrophotometer (JASCO V-550, Japan).

Membrane degradation in isolated hepatocytes in terms of Lactate Dehydrogenase (LDH) Release: The membrane degradation in isolated hepatocytes was monitored according to the method described by Moldeus et al[12]. An aliquot of cell suspension was centrifuged at 200 g for 5 minutes, 0.02 ml of cell free medium was mixed with 1.5 ml of solution containing Tris – HCl (100 mM), NADH (0.2 mM) and pyruvate (1.4 mM) and change in absorbance at 340 nm was recorded. A separate aliquot of cell suspension (0.25 ml) was treated with 0.25 ml of Triton X–100 for the measurement of total LDH activity.

Lipid Peroxidation (LPO) Assay: MDA liberated during the course of LPO was estimated by the thiobarbituric acid (TBA) method of Okhawa et al[14]. MDA is the most abundant individual aldehyde resulting from LPO. It was determined by TBA reaction. To the sample plate containing 0.1 ml distilled water, 0.75 ml of 20% acetic acid, 0.2 ml SDS, 0.75 ml of 1% TBA and 0.1 ml of cell lysate was added. In blank there was only 0.1 ml distilled water instead of cell lysate. The solutions were mixed and heated in water bath for 60 min at 95ºC. After cooling followed by proper mixing the centrifugation was done at 1000 g for 15 min. The Optical Density (O.D.) of supernatant was read at 535 nm spectrophotometrically and was expressed as n moles MDA / 10,000 cells.

Ca^{2+} ATPase activity in isolated hepatocytes: Ca^{2+} ATPase was assayed in hepatocytes according to the method of Desaich et al[15]. Ca^{2+}ATPase is a plasma membrane bound enzyme that expels Ca^{2+} at the expense of hydrolysis of ATP to ADP and inorganic phosphate. The inorganic phosphate released by the action of Ca^{2+}ATPase is estimated colorimetrically. The Ca^{2+} ATPase was assayed in a reaction mixture (1.5 ml) containing 40mM Tris HCl buffer (pH 7.5), 5mM $MgCl_2$, 0.05mM $CaCl_2$, appropriate amount of hepatocyte membranes and 2.5mM ATP. The Ca^{2+}ATPase activity was determined in presence of 1mM EGTA and this was subtracted from total Ca^{2+}ATPase activity in order to obtain net Ca^{2+}ATPase activity. The reaction mixture was incubated at 37^0C for15 min. and afterwards 0.1 ml of 50% w/v/TCA was added to stop the reaction. The content was centrifuged at 3000 g for 10 min. and estimated in the supernatant by the method of Fiske and Subbrarow[16].

Statistical Analysis: The data are the mean ± SD of three separate observations employing one way Analysis-of-Variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test for comparison between groups. Values having $p<0.05$ were considered significant.

Results and Discussion

Effect of APAP was observed in terms of cell damage as determined by MTT reduction assay which identifies dead cells with damaged membranes. The MTT reduction assay results obtained at various APAP concentrations and LDH leakage were used as an indicator of cell membrane damage and hepatocytes viability. Present experiments showed that in absence of acetaminophen, the viability of hepatocytes in term of MTT reduction was 96%. A decrease in viability of hepatocytes was observed at increasing concentrations of APAP. At 5mM concentration of APAP, 59% MTT reduction was recorded whereas at 40mM the reduction declined to 8% (figure 1). The observation indicated substantial deterioration in the functional capability of the cell. The measurement of the oxidation potential of an artificial substrate by mitochondrial and extra-mitochondrial dehydrogenases in MTT assay, reflects overall functional cell viability[17, 18]. APAP caused a progressive impairment of dehydrogenase activity at the time of severe

cellular GSH depletion. These findings are consistent with observations *in vivo*, where the depletion of cytosolic and mitochondrial GSH after APAP overdose is followed by an increase of the GSSG-to-GSH ratio and impairment of the mitochondrial respiration and ATP depletion[19-21]. Mitochondrial proteins are preferred targets of NAPQI[22]. The importance of mitochondria is supported by the fact that APAP and the nonhepatotoxic isomer 3'-hydroxyacetanilide show similar overall protein binding, but there is substantially more covalent binding of mitochondrial proteins after APAP[23]. In addition, exposure of rat hepatocytes to NAPQI simulated the mitochondrial dysfunction observed with APAP[24]. Although mitochondrial GSH depletion and increased Ca^{2+} uptake may also contribute to mitochondrial dysfunction, these data suggest that covalent binding of NAPQI to mitochondrial proteins may play an important role in causing impaired mitochondrial respiration. Our results indicate the release of ROS may be due to the impaired mitochondrial function. Moreover, at hepatotoxic doses of APAP early ultra structural changes in mitochondria are observed whereas some minor changes also are seen with sub-toxic doses[25]. Most importantly, all these events occur several hours before the onset of cell membrane permeability indicated by trypan blue uptake and LDH release[26].

Biological protection conferred by several plants depends on the presence of components with antioxidant activity, as is the case of *Curcuma longa*[27]. One percent of turmeric in the diet was recommended by obtain cellular protective effects[28]. It was observed that turmeric does not affect body weight gain or food ingestion in rats, which is in accordance with the literature[29]. Turmeric, however, presented significant antioxidant activity, the inhibition of lipid peroxidation probably being due to arrest of ROS[30]. On the other hand, it was interesting to examine the protective effect of turmeric on cells challenged by the presence of APAP a well known hepatotoxic compound. Hepatocytes primary culture is adequate to study the effect of APAP hepatotoxicity[31].

The primary toxicity of APAP is the result of drug metabolism in both the liver and extrahepatic tissues[32]. It has been suggested that LPO might be a contributing factor to the development of renal toxicity. It is likely that the action of CMN in reducing the membrane damage is partially related to its ability to scavenge LPO initiating agents[33]. In the present study, we also recorded a significant increase in the MDA levels in the hepatic tissue of rats treated with APAP alone as compared with the control group. On treatment with CMN at the recommended concentration the MTT reduction improved to 52% indicating an improvement in the hepatocyte viability. The dose response graph is shown in (figure 1).

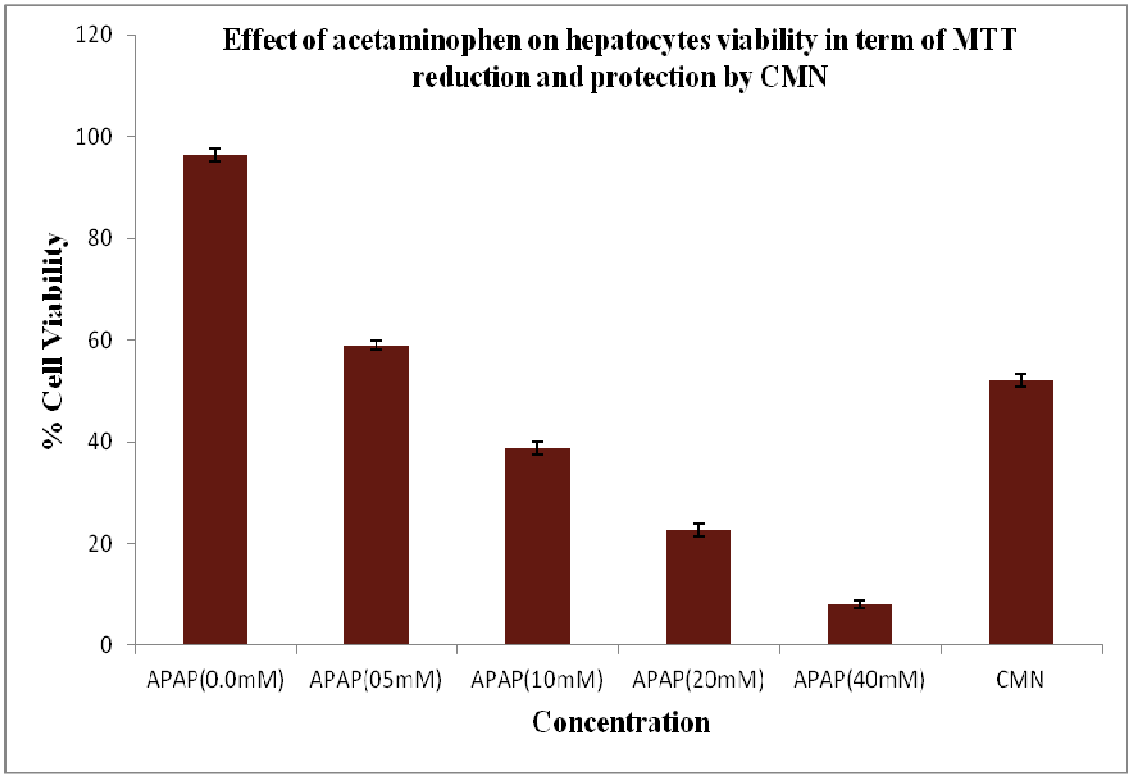

Figure-1
Effect of varying concentration of acetaminophen (upto 40 mM) on hepatocytes viability measured in terms of MTT reduction and protection conferred by CMN

APAP induced hepatotoxicity was observed in terms of LDH leakage. LDH release quantification is used as an parameter to assess cytotoxicity, cell membrane damage and hepatocytes viability. Intracellular LDH release was evaluated as a result of breakdown of plasma membrane and alteration of its permeability. A concentration dependent increase in LDH release was observed. In absence of acetaminophen, the LDH release was 2% whereas at 5mM concentration, the LDH release recorded was 41%. On increasing the concentration further to 40 mM the LDH release increased significantly to 83% (figure 2). The effect of CMN at recommended dose was used and remarkable decrease to 63% in the release of LDH was observed indicating a beneficial effect of curcumin on cell viability.

Analysis of LPO levels by TBARS reaction showed a significant ($P<0.001$) increase in the MDA level in APAP treated rat hepatocytes. The increase in LPO level in APAP treated hepatocytes indicated enhanced tissue damage as a consequence of failure of antioxidant defense mechanism to prevent formation of excessive free radicals. In the absence of APAP the MDA level was ~0.3nM/mg of protein whereas on increasing the APAP concentration to 40mM the level increased to 0.67 nM/mg of protein indicating the damage caused to the cell membrane (figure 3). APAP induced hepatic injury was due to an increase in MDA level, which is in agreement with the previous studies[34]. The protection conferred by CMN was assessed at recommended concentration and significant improvement was recorded. The MDA level decreased to 0.42 nM/mg of protein suggesting improvement in hepatocyte viability. APAP on overdose elevated the production of LPO. Thus an increase in the liver MDA levels suggests an increase in the degree of LPO, a well known mechanism of liver damage. In addition, the extensive LPO results in membrane disorganization by peroxidizing the highly unsaturated fatty acids, which in turn alters the ratio of polyunsaturated fatty to other fatty acids leading to decrease in membrane fluidity, which may be sufficient to cause cell death. In CMN pre-treated rats a significant decrease in the level of lipid peroxides suggests that CMN may have the ability to protect the liver from APAP induced free radical injury.

APAP hepatotoxicity was further evaluated in terms of Ca^{2+} ATPase activity. In the absence of APAP Ca^{2+}ATPase activity was 19.21 mM ATP hydrolyzed/min/mg of protein and significant decrease in the activity to 8.37 mM ATP hydrolyzed/min/mg of protein was recorded at higher concentration of APAP (40 mM) (figure 4). This might be due to the release of these enzymes, from the cytoplasm, into the blood circulation rapidly after rupture of the plasma membrane and cellular damage. The treatment with CMN showed significant improvement in Ca^{2+} ATPase activity (10.58 mM ATP hydrolyzed/min/mg of protein). Decrease in Ca^{2+} ATPase enzyme activity is a sensitive index of hepatocellular damage.

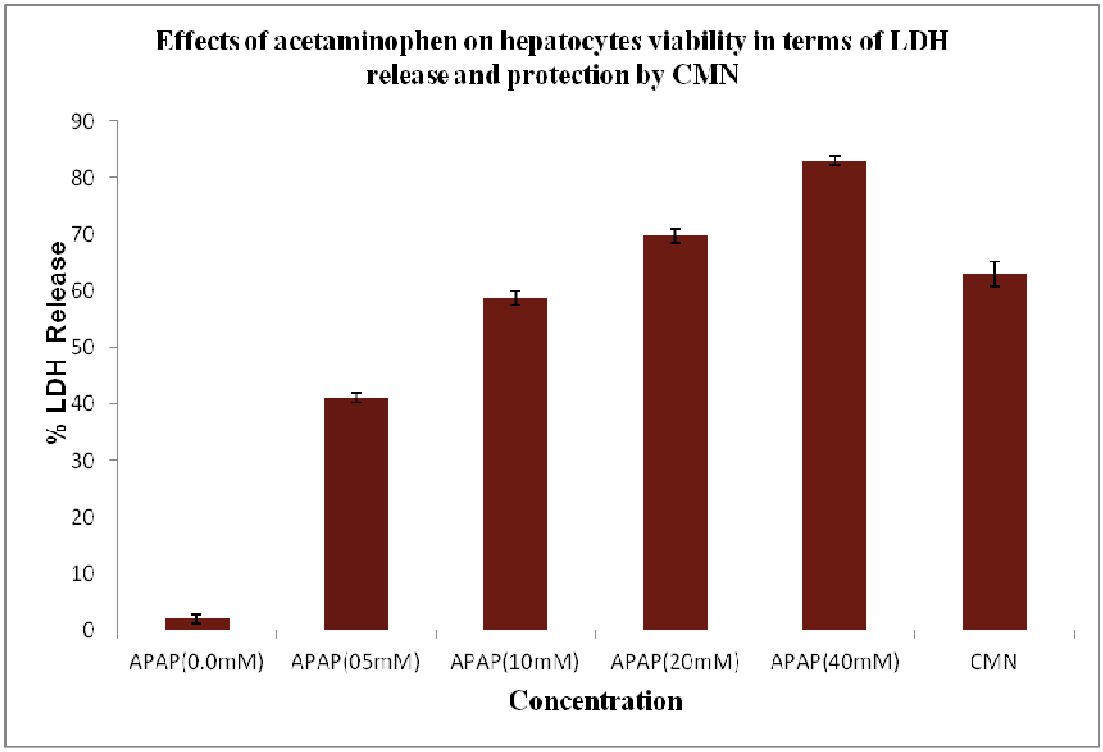

Figure-2
Effect of varying concentration of acetaminophen (upto 40 mM) on hepatocytes viability measured in terms LDH release and protection conferred by CMN

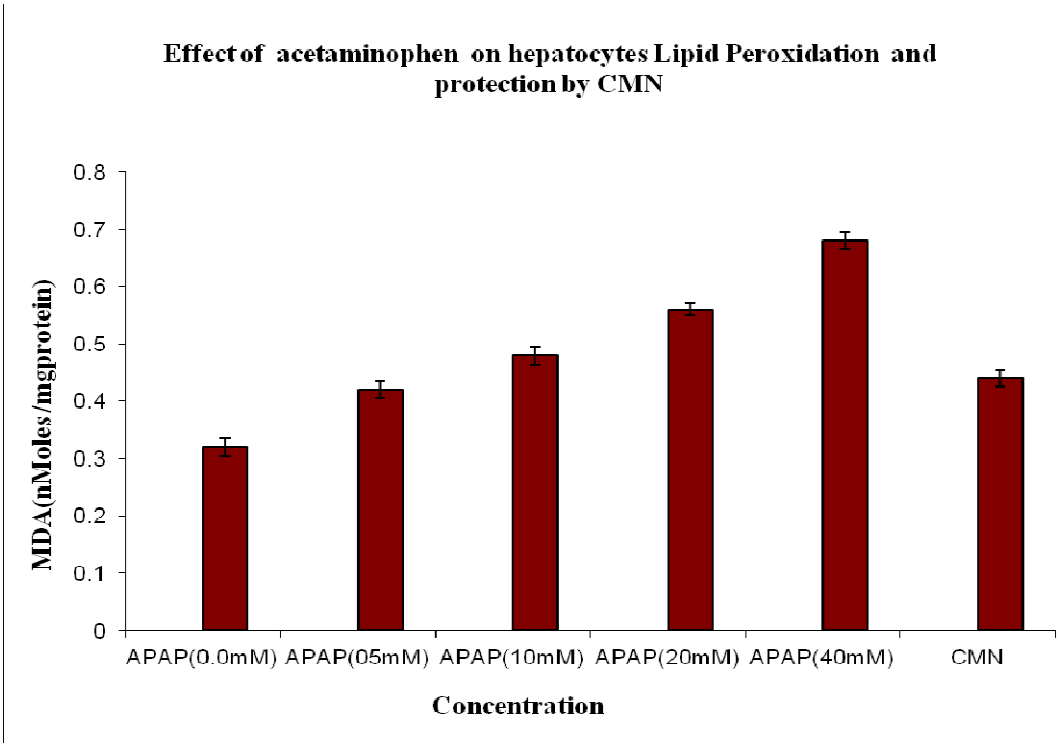

Figure-3
Effect of varying concentration of acetaminophen (upto 40 mM) on hepatocytes viability measured in terms lipid peroxidation and protection conferred by CMN

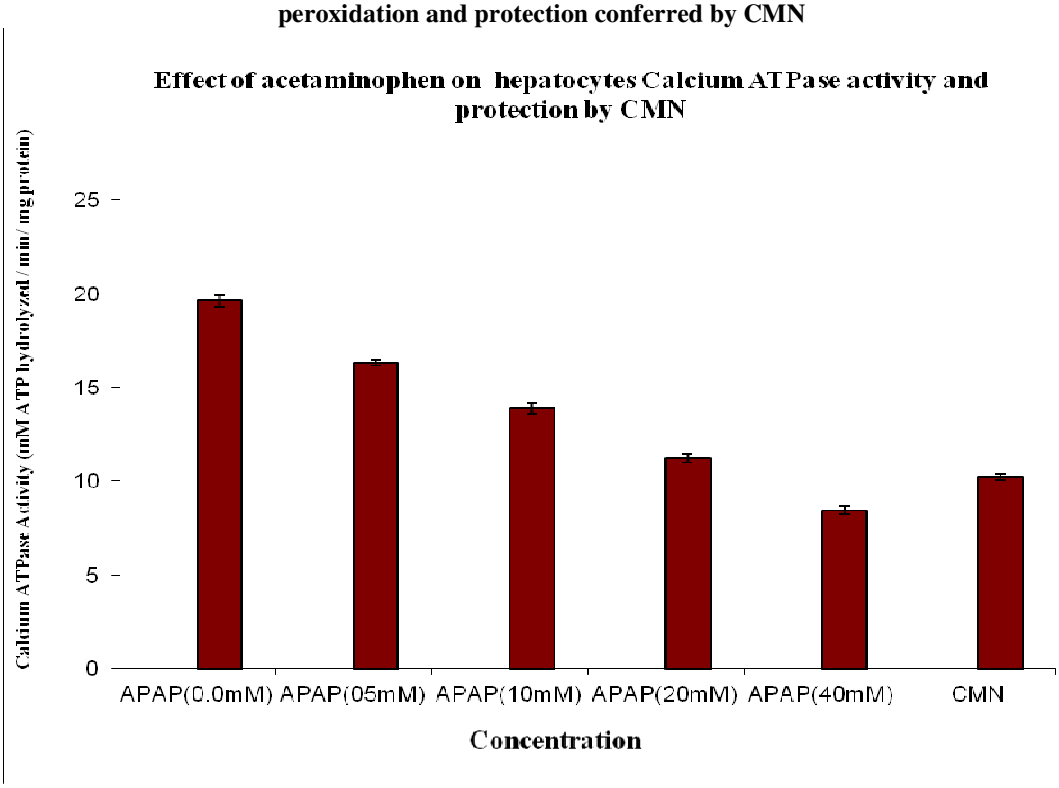

Figure-4
Effect of varying concentration of acetaminophen (upto 40 mM) on hepatocytes calcium ATPase activity and protection conferred by CMN

The results reveal that significantly higher levels of LDH release, LPO and lower level of Ca^{2+}ATPase was observed in APAP treated rats when compared to control group. These oxidants produce toxicity possibly by protein oxidation, enzyme inactivation and damage to cell membrane via LPO and production of lipid aldehyde as MDA. It is clear from the above results that APAP is able to generate oxidative stress in hepatocytes which is responsible for decreased cell viability as it exerts it toxic effects by acting on a wide variety of sites. Biological effects of t-BHP have been studied as a model for oxidative cellular injury and perhaps APAP may also works as a hepatotoxin that can deplete soluble and protein thiols and disrupt Ca^{2+} homeostasis[35].

CMN is a major yellow pigment in turmeric (the ground rhizome of *Curcuma longa* Linn), which is widely used as a spice and a natural colouring agent in several foods such as curry, mustard and potato chips as well as in cosmetics and drugs[36, 37]. It represents a class of antioxidants and anticancer agents with a strong potential in down regulating the generation of ROS [38,39]. CMN administration has been reported to prevent hepatic lesions in streptotocin diabetic rats and to protect against oxidative stress in hepatic cell lines[40,41]. It also prevents or attenuates nephrotoxicity caused by cisplatin and adriamycine[42,43]. In spite of its efficacy and safety, CMN has not yet been approved as a therapeutic agent. The poor aqueous solubility, relatively low bioavailability, and intense staining colour of CMN have been highlighted as major problems; and consequently search for a ‘‘super curcumin’’ without these problems and with efficacy equal to or better than that of curcumin is ongoing.

The primary toxicity of APAP is the result of drug metabolism in both the liver and extrahepatic tissues[32]. At therapeutic doses, APAP is metabolised via glucuronidation and sulfation reactions occurring primarily in the liver which result in the water-soluble metabolites that are excreted via the kidney. The metabolic conversion of APAP by the microsomal P-450 enzyme system results in the generation of a highly reactive intermediate, viz., N acetyl-p-benzoquinone imine (NAPQI). This metabolite is kept in check by reduced using GSH[44]. APAP-induced hepatotoxicity may possibly be due to the conversion of APAP to a highly reactive metabolite. In cases where consumption of APAP is high, there is severe GSH depletion as well as massive production of metabolites, which aggravates the toxicity, leaving large amounts of reactive metabolite in unbound state. Consequently, these intermediates then form covalent bindings with macromolecules on cellular protein[44]. A major fall out of this process is disruption of homeostasis and initiation of apoptosis (programmed cell death), leading to tissue necrosis and ultimately culminates to organ dysfunction. Thus, concentration of intracellular GSH is the key determinant of the extent of APAP-induced hepatic injury. As a result, interest has been focused on compounds like CMN that act as antioxidants and are capable of stimulating GSH synthesis. It is likely that the reduction in membrane damage is partially related to the ability of CMN to scavenge LPO initiating agents[33]. In this study, we also observed a significant increase in the MDA levels in the hepatic tissue of rats treated with APAP alone compared with the control. APAP-induced hepatic damage is consistent with acute tubular necrosis. In a report histopathological examination shows a clear evidence of hepatotoxicity following the overdose administration of APAP[45]. The preventive effect of CMN on the hepatotoxicity induced by APAP possibly depends on its ability to eliminate the hydroxyl radical, superoxide radical, singlet oxygen, nitrogen dioxide and NO[28,46-48]. It has also been demonstrated that CMN inhibits the generation of superoxide radical[49].

Conclusion

it can be concluded that APAP administration caused a significant down regulation of endogenous antioxidant profile which was effectively reverted with CMN treatment.

Acknowledgement

Financial assistance from UP Council of Science and Technology, Lucknow, U.P., India vide grant no. CST/SERPD/D-1949 is gratefully acknowledged. The grant received from Department of Science and Technology under FIST programme is thankfully acknowledged.

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