International Standard Serial Number: 0976-139X

Deccan J. of Pharmacology 1(2): April-June 2010

DECCAN PHARMA JOURNAL SERIES

ARMS Online Publications

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PROTECTIVE EFFECT OF RESVERATROL AGAINST CARBON TETRACHLORIDE INDUCED OXIDATIVE STRESS IN RAT LIVER

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ABSTRACT

The purpose of this study was to investigate the role of free radicals in carbon tetrachloride induced hepatic damage and elucidates the mechanism of resveratrol. To achieve this resveratrol was administered (100mg, 200mg/kgbody weight) orally for 8 weeks and liver damage was induced by daily administration of carbon tetrachloride (0.4g/kg/ body weight) intraperitoneally, appropriate controls were performed, After sacrifice the animals liver samples were taken for histological examination, blood samples were collected for various biochemical examination, TNF-α, IL-6 and total antioxidant capacity were assayed in serum sample. Carbon tetrachloride administration caused a significant decrease in reduced glutathione (GSH) levels in serum of different groups of rats. Which was accompanied with significant increases the pro-inflammatory mediators mainly TNF-α, IL-6 and induces lipid peroxidation. Antioxidant and free radical scavenging activity of resveratrol treatment reversed all these alterations induced by carbon tetrachloride.

Key words: Resveratrol, Carbon tetrachloride (CCl_4), Tumor Necrosis Factor alpha (TNFα), Lnterleukin-6 (IL-6)

1. INTRODUCTION

Liver intoxication has increased as a result of exposure to high levels of environmental toxins, it has an important role in detoxification. Liver is a primary target organ for many toxic chemicals. Inflammatory processes participate in a number of pathological (necrosis and fibrosis), protective and repair events following exposure to hepatotoxic chemicals[1] and other inflammatory diseases are responsible for producing the mediators (cytokines) which can affect the liver damage[2, 3].

Carbon tetrachloride (CCl_4) is a hepatotoxin, causing liver necrosis, fibrosis and cirrhosis when administered sequentially. Involvement of lipid peroxidation in carbon tetrachloride induced hepatoxicity is widely accepted, although covalent binding of the compound to cellular macromolecules may also contribute to the damage. It has been proposed that Kupffer cells may be involved in the hepatotoxicity of carbon tetrachloride, as a source of cytotoxic factors, such as active oxygen species, leading to hepatocellular damage as well as playing a role in the regeneration of hepatic tissue [4].

Biological free radical reactions are involved in the reduction of molecular oxygen to yield reactive oxygen species such as the superoxide anion and hydroxyl radical. Various diseases such as liver disease and diabetes may reduce the number of these radicals, and reactive oxygen species (ROS) may be essential for cellular functions such as ingestion of bacteria and redox regulation of signal transduction. However, ROS are generally harmful agents that cause considerable damage to cellular components such as lipids, proteins, and DNA. Moreover, lipid peroxides promote the formation of additional free radicals in a type of chain reaction[5].

A number of plants contain substances that can protect or treat hepatic injury. Antioxidant action plays an important role in protection against carbon tetrachloride-induced liver injury[6]. Resveratrol (trans-3, 5, 4′-trihidroxystilbene) (Figure 1) is a nonflavonoid polyphenolic compound that is found in a great variety of plant species, some of which are part of the human diet. These include peanuts, grapes and red wines[7]. It exists in the cis and trans conformation. The trans to cis isomerisation is facilitated by UV light. Many in vivo and in vitro studies describe the different effects of resveratrol in which this compound possess several pharmacological activities including anticancer, cardio protective, neuroprotective, antioxidant, anti-inflammatory[8]. It has also having potent antidiabetic, antinociceptive, antiteratogenic, anti asthmatic activity. Reports are also suggested that it has an effective role in osteoarthritis, glaucoma and pancreatitis[9].

Further reports are suggested that resveratrol protects against oxidative stress in cholestasis and exerts hepatoprotective activity against hepatotoxicants like ethanol, thioacetamide induced acute and chronic liver damage in rodents [10, 11]. Ex vivo production of inflammatory and anti inflammatory cytokines TNF α, IL-1β and IL-6 are stimulated by lipopolysaccharides (LPS) can be suppressed by immunomodulatory effect of trans resveratrol in a concentration dependent manner and play an important role in disease conditions and suppresses the overproduction of inflammatory cytokines[12].

This study was designed to investigate the effect of resveratrol on antioxidant defense systems, serum biochemical parameters and histological changes of liver in carbon tetrachloride induced rat hepatic injury model.

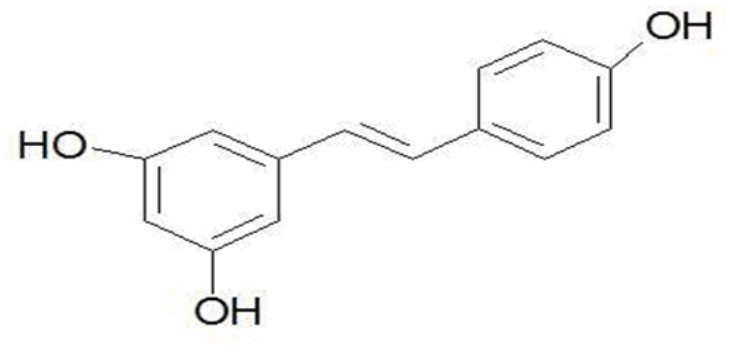

Figure 1

2. MATERIALS AND METHODS

2.1 Chemicals

Resveratrol, Carbon tetrachloride (CCl_4), Biotinylated rabbit anti mouse IgG, Streptavidin peroxidase, 3,3 diaminobenzidine tetrahydro chloride (DAB) were obtained from sigma chemical co (St.Louis, MO, USA). The rabbit anti mouse TNF-α and IL-6 antibody was purchased from Anaspec Inc. (San Jose, CA). All other chemicals and reagents were used of analytical grade purchased purest form available from local firms.

2.2. Animals

Pathogen-free male wister rats, initially weighing 100-150g, were procured from National Institute of Nutrition (NIN, Hyderabad, India). Rats were maintained in an air conditioned room (temperature 18 ± 2ºC, relative humidity 40-70%, and a 12:12 h light-dark cycle) and housed in solid bottom polypropylene cages (four animals / cage) bedding for one week before starting the experiment. The animals were fed with a semi purified basal diet (Table 1) and demineralized drinking water *ad libitum*. All the experimental procedures were conducted in conformity with "Institutional Animal Ethical Committee" ["Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA Regn. No. 1126/bc/07CPCSEA) India] for the care and use of laboratory animals were strictly followed throughout the experiment.

Table 1. Composition of food mixture

Ingredient	Amount (g /kg diet)
Wheat flour (*Triticum aestivum*)*	500
gram (*Cicer artetinum*)	150
Maize dust (*Zea mays*)	150
Milk powder (spray dried)	150
Macromineral mix§	49.75
Micromineral mix¶	0.25

*Wheat flour, Bengal gram, maize and spray dried milk contain sufficient amounts of vitamins and fatty acids and no extra vitamin mixtures were added to the food

§Macromineral mix provided (g / kg food): $CaCO_3$, 16.7; $NaHPO_4$, 13.7; NaCl, 6; KCl, 12; MgO, 17.5.

¶Micromineral mix provided (mg / kg food): $FeSO_4.7H_2O$, 216; $CuSO_4$, 20; $Ca(IO_3)_2$, 1.3; MnO, 5; $CoCl_2.6H_2O$, 1.9; Na_2SeO_3, 0.30; $CrCl_3.8H_2O$, 4; $Na_2Mo_4.2H_2O$, 1.5.

2.3 Experimental groups

After acclimatization, rats were ramdomly divided into four groups each consisting of 10 rats shown in figure 2.

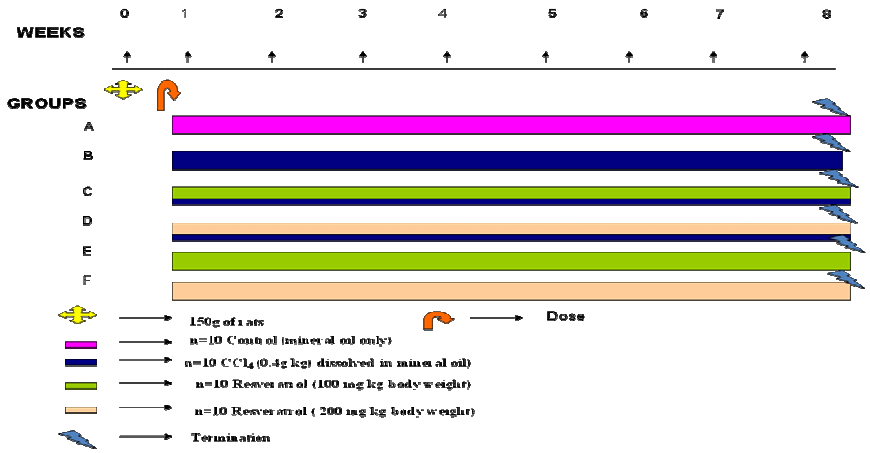

Figure 2

Group A: Normal control: Animals received vehicle only (0.1ml of mineral oil) p.o.

Group B: CCl_4 control: Animal received CCl_4 (0.4g/kg dissolved in mineral oil) by intraperitonially.

Group C: Treatment group: Animal received resveratrol at a concentration of 100mg / kg orally along with CCl_4 (0.4g/kg).

Group D: Treatment group: Animal received resveratrol at a concentration of 200mg / kg orally along with CCl_4 (0.4g/kg).

Experiment was conducted up to 8 weeks at the end of this period animals were sacrificed 72 h after the last dose of resveratrol or carbon tetrachloride under light ether anesthesia; blood was collected by retroarbitary vein, separate the serum by centrifuged at 4000rpm for 60min and stored at -20ºC until parameters was analyzed and the livers were isolated for histopathological study.

2.4 Biochemical analysis

Serum Glutamate Pyruvate Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT) Alkaline Phosphatase (ALP), Bilirubin, total protein level (TP) and albumin level (ALB) were estimated by using commercially available biochemical estimation kits (Ensure Biotech Pvt. Ltd, Hyderabad, India), determined by spectrophotometrically using an automated analyzer[13].

2.5 Assessment of Lipid peroxidation

The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation using UV- visible spectrophotometer at 532 nm. Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 m^{-1} cm^{-1} and the results are expressed as nmol MDA/g tissue[14].

2.6 Total antioxidant and Glutathione assay

The total antioxidant capacity in plasma was measured by using colorimetric test system (Systronic 2202, Ahmedabad, India) according to the instructions provided by the manufacturer.

Tissue samples were homogenized with ice cold trichloracetic acid (1g tissue plus 10 ml 10%TCA) in an Ultra Turrax tissue homogenizer. It was performed according to Ellman procedure. Its levels were calculated using an extinction coefficient of 1.36×10^4 m^{-1} cm^{-1}. And the results are expressed as µmol GSH/g tissue.

2.7 Histopathology

Eight weeks after the CCl_4 or vehicle treatment animals from each group were randomly selected, a portion of the liver was excised from ether anaesthetized rat fixed in 10% formalin and processed for histopathological studies. The tissues were dehydrated through 70, 90 and 100% alcohol and embedded in low melting point paraffin wax. Section of 5 µm thickness were cut and placed serially on glass slide. The sections were deparaffinised in xylene and rehydrated through 100, 90 and 70% alcohol. Each liver tissue was stained with hematoxylin and eosin for histological evaluation using light microscopy. The histological slides were coded so that the particular sample identity was known to the individual making the assessment.

2.8 Enzyme linked Immunosorbent assay (ELISA)

Serum levels of tumor necrosis factor alpha (TNF α), interleukin-6 (IL-6) were quantified according to the manufacturer's instructions and guidelines using enzyme linked immunosorbent assay (ELISA) kits specific for the previously mentioned rats cytokines (Anaspec Inc. San Jose, CA). These particular assay kid was selected because of their high degree of sensitivity, specificity, inter and intraassay precision and small amount of plasma sample required to conduct the assay.

2.9 Statistical analysis

Results are presented as mean value ± S.E.M. Comparisons are carried out by analysis of variance followed by post test (Dunnett's test) as appropriate using graph pad prism. Differences are carried out statistically significant, when $P<0.01$, $P<0.05$, $P<0.001$.

3. RESULTS

3.1 Biochemical levels

Administration of carbon tetrachloride to rats caused significant increase in serum enzymes such as SGPT, SGOT, ALP and bilirubin and decrease in serum total protein level (TP) and albumin level (ALB) compare to normal control rats shown in (Table 2). CCl_4 induced rats showed significant difference ($p<0.05$) compare to normal control rats. When rats treated with resveratrol 100mg/kg orally exhibited a significant $P<0.01$ hepatoprotective effect by reducing the serum bilirubin level and enzyme levels and increasing the protein levels compare to CCl_4 induced rats. Resveratrol 200mg/kg treated rats showed more decreased the levels of serum enzymes, and increased the levels of total protein and albumin compare to resveratrol 100mg/kg and CCl_4 control rats. This group showed statistically significant $P<0.001$ with CCl_4 control rats. CCl_4 control rats were exhibits significant difference ($P<0.05$) with resveratrol 100mg/kg, $P<0.01$ and 200mg/kg. Administration of resveratrol (100mg, 200mg/kg) attenuated the increased levels of the serum enzymes produced by CCl_4 and caused a subsequent recovery towards normalization.

Table 2. Effect of Resveratrol on the Concentration of Enzymes (SGPT, SGPT, ALP), Bilirubin, Total protein (TP), Albumin (ALB)

Groups	Doses (mg/kg)	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L)	Bilirubin (mg%)	TP (gm%)	ALB (gm%)
Normal control	-	13.2±0.4*	38.2±0.7*	83.63±1.6*	0.26±0.04*	7.28± 0.16*	3.89± 0.14*
CCl_4 control (0.4g/kg)	0.4	150±1.9a,b	167.6±1.6a,b	115.2±1.9a,b	1.29±0.05a,b	5.59± 0.25a,b	1.47± 0.15a,b
Resveratrol (100mg/kg)+CCl_4	100	98.6±1.4**	109.8±1.3**	101.2±1.3**	0.79±0.04**	6.63± 0.42**	1.96±0.12**
Resveratrol (200mg/kg)+CCl_4	200	88.6±1.2***	62.6± 1.2***	95.3±1.9***	0.65±0.03***	6.92±0.36***	2.9±0.05***

Values are mean ± S.E.M of 10 rats from each group
* $P<0.05$, significant differences compared to CCl_4 control rats
** $P<0.01$, significant differences compared to CCl_4 control rats
*** $P<0.001$, significant differences compared to CCl_4 control rats
a $P<0.05$, insignificant differences compared to resveratrol 100mg/kg treated rats
b $P<0.01$, insignificant differences compared to resveratrol 200mg/kg treated rats

3.2 Lipid peroxidation

The degree of lipid peroxidation was measured as a one of its end products, malondialdehyde (MDA). CCl_4 intoxication significantly increased the liver content of MDA; treatment with resveratrol 100mg/kg, 200mg/kg completely prevented increased levels of MDA, degree of lipid peroxidation (Figure 3).

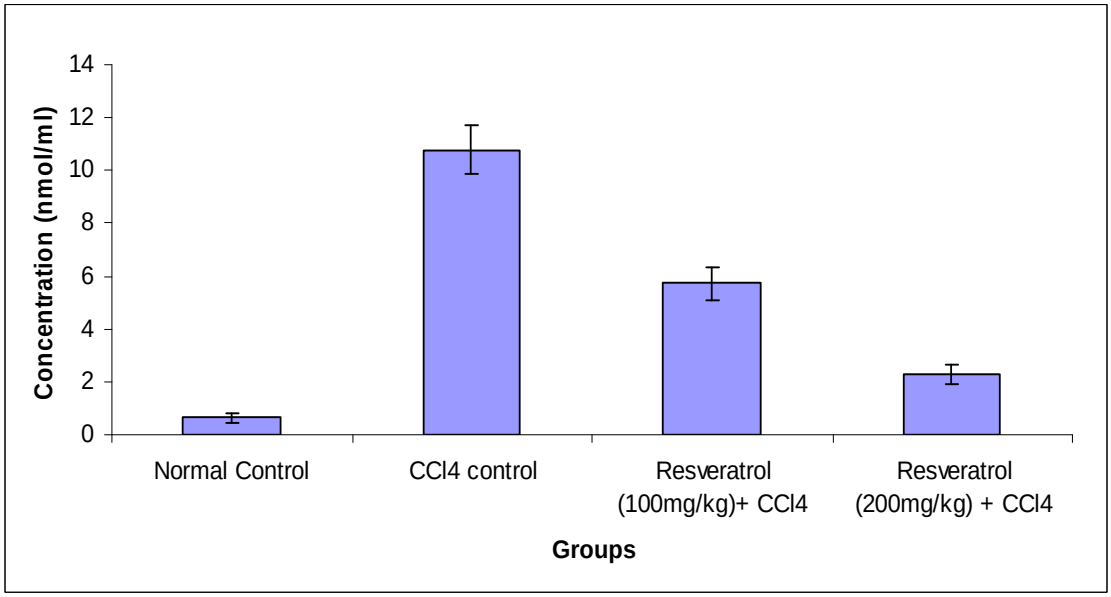

Figure 3

Normal control group showed significant difference P<0.01 with CCl_4 control. Resveratrol 100mg/kg, 200mg/kg showed statistical significant P<0.05 with CCl_4 control group and also Group IV shows significant P<0.05 with Group III shown in (Table 3).

Table 3. Effect of resveratrol on TBARS, Total antioxidant, Glutathione.

Groups	TBARS (nmol/ml)	Total antioxidant (µmol/ml)	Glutathione (µmol/ml)
Normal control	0.64±0.2[a]	60.7± 0.9[a]	93±1.2[a]
CCl_4 control	10.79±0.9	25.2± 1.2	38±0.4
Resveratrol (100mg/kg)+ CCl_4	5.71±0.6[b]	113.7±1.4[b]	58±0.6[b]
Resveratrol (200mg/kg)+ CCl_4	2.28±0.4[c,d]	171.2±1.6[c,d]	78±0.8[c,d]

Values are mean ± S.E.M of 10 rats from each group
[a] $P<0.01$, significant differences compared to CCl_4 control rats
[b] $P<0.05$, significant differences compared to CCl_4 control rats
[c] $P<0.05$, significant differences compared to CCl_4 control rats
[d] $P<0.05$, insignificant differences compared to resveratrol 100mg/kg treated rats

3.3 Total antioxidant and Glutathion levels

Table 3 shown total antioxidant levels are very high in resveratrol 200mg/kg because it acts as a potent antioxidant agent. High levels free radicals are generated in this group of rats for this reason very less amount of total antioxidant, sometimes complete absence were seen in CCl_4 control rats. Control rats

showed significant difference P<0.01 with CCl_4 rats. Resveratrol groups showed P<0.05 significance with CCl_4 group (Figure 4).

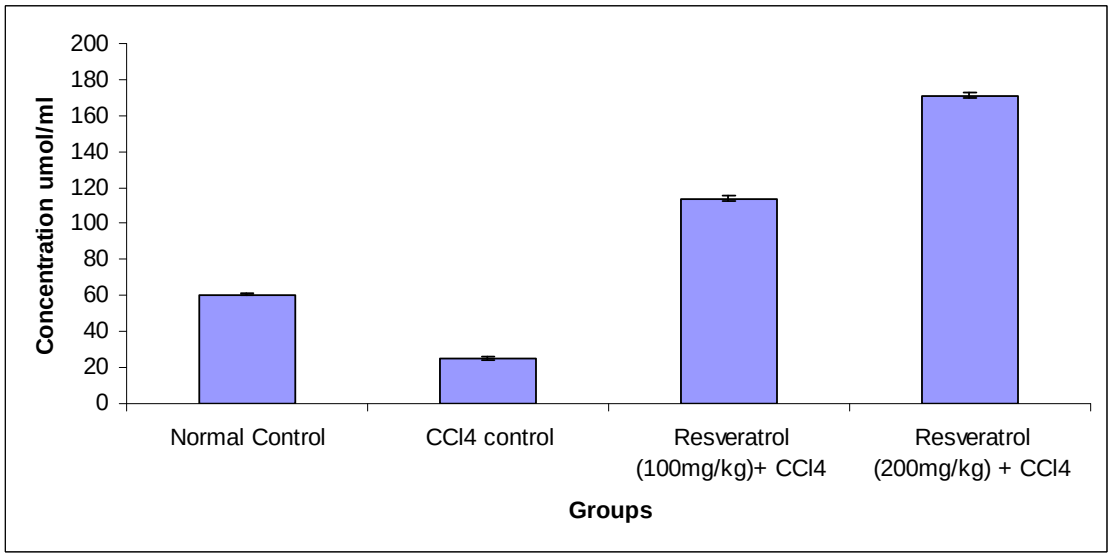

Figure 4

Similarly glutathione levels also very less in CCl_4 control group shown in (table 3) This levels are gradually increased with resveratrol in resveratrol treated groups and also showed statistical significance P<0.05 with CCl_4 control (Figure 5).

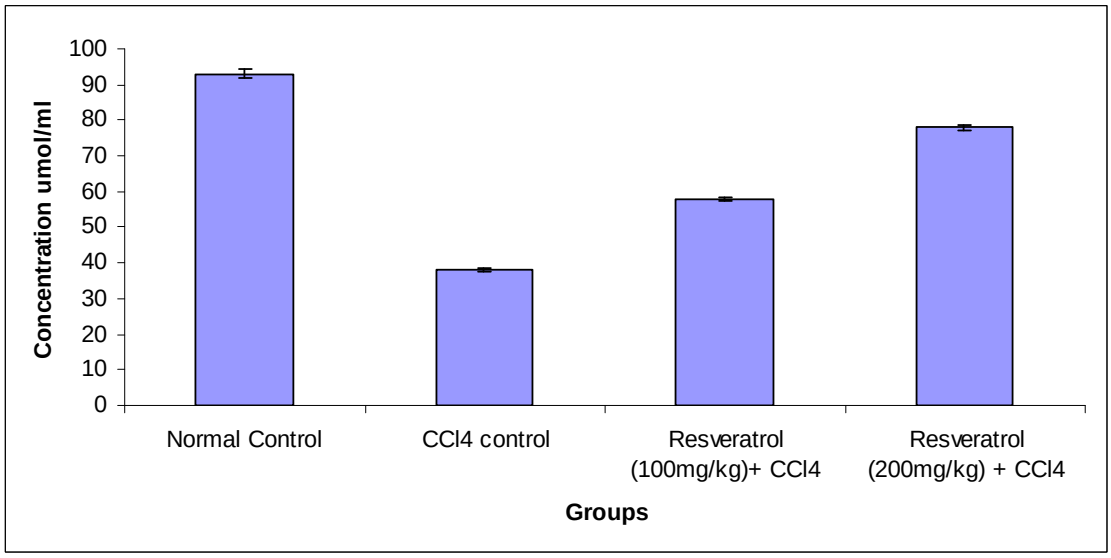

Figure 5

3.4 Histopathological examination

Histopathological analysis of liver sections from various experimental groups of animals was depicted in (Figure 6). The purpose of histological staining method by hematoxylin and eosin is visualized and differentiate between tissue components in normal and pathological condition. Identification and conformation of histopathological change during hepatocellular damage (necrosis) and the protective effect of resveratrol against CCl_4 induced toxicity was carried out in (Figure 6a). The histopathology of liver from

the normal control (Group A) showed a normal arrangement of hepatocytes with clearly visible nucei, central vein shown in (Figure 6a). Intraperitoneal injection of CCl_4 at a dose of 0.4g/kg body weight (Group II) showed extensive centrilobular necrosis around the central vein of liver and dilation of the central vein with few regenerative areas of hepatocytes around the central vein and distal part of liver was observed in (Figure 6b). The magnitude of dilation of the central vein was prominent but very less in comparison with the CCl_4 control (Figure 6c). The liver sections from the group IV showed good protection of hepatocellular necrosis with a poorly dilated central vein and regular arrangement of hepatocytes around the central vein and also the distal part of the liver from the central vein was observed along with a scattered regenerative zone of hepatocytes, very few necrotic zones were prominent (Figure 6d)

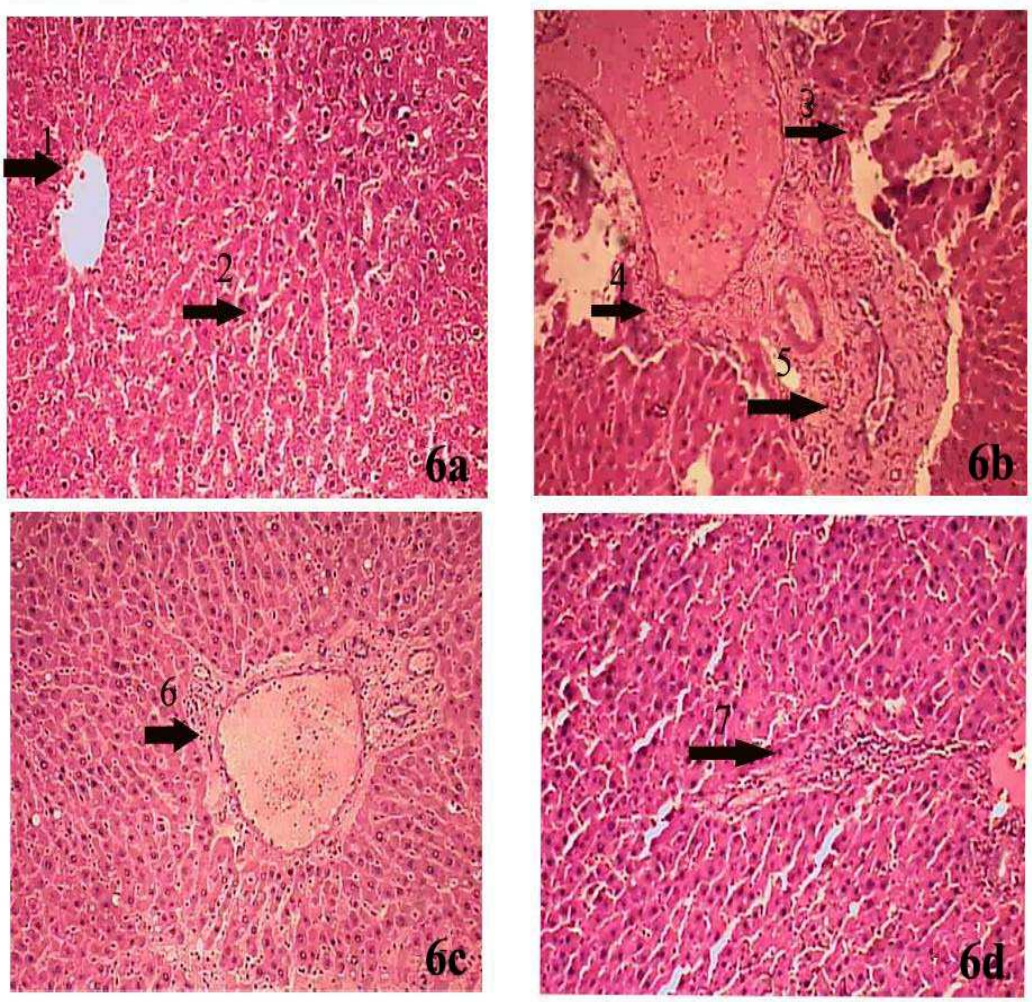

Figure 6

3.5 Serum levels of TNF α, IL-6

As shown in table 4, level of circulating TNF α, IL-6 were higher in CCl_4 control than normal control rats (P<0.01). however, the values of these two cytokines were significantly reduced after treatment of resveratrol 100mglkg group (P<0.05). resveratrol 100mglkg group (P<0.001) compare to CCl_4 control.

4. DISCUSSION

Liver is an important organ actively involved in metabolic functions, is a frequent target of number of toxicant. Carbon tetrachloride is an important xenobiotic, is a widely used chemical to induce liver damage in experimental animals and its toxicity has been studied extensively. Many clinical and experimental data provided carbon tetrachloride is a classical hepatotoxicant causing liver cirrhosis, fibrosis, and necrosis. High levels of mixed function oxidases in the hepatocytes especially in the centrilobular region are responsible for producing highly reactive trichloromethyl free radical, which initiate lipid peroxidation and causing centrilobular necrosis. Again mixed function oxidizes covalently bind with the cellular macromolecules like kupffer cells, hepatic stellates and sinusoid endothelial cells resulting in production of proinflammatory cytokines causes infiltration of neutrophiles and monocytes into the damaged organ . These cells produced additional cytokines such as IL-6, TNF-α, TGF-β, eNOS as well as reactive oxygen species and reactive nitrogen species that causes injury in liver[15]. TNF-α, IL-6 are considered as a major hepatotoxic mediators in several experimental modules of liver injuries[16].

Resveratrol protects partially from oxidative stress, necrosis and cholestasis induced CCl_4 intoxication. In rats as in humans resveratrol is rapidly absorbed, metabolized to glucuronides or sulphate conjugates and distributed to various organs, being the primary targets the liver, heart and kidneys. Particularly the liver is responsible for a high accumulation of resveratrol after ingestion.

The resulting hepatic injury was characterized by leakage of cellular enzymes into the blood stream and by centrilobular necrosis. Elevation of enzymes in the blood is most often associated with liver injury or disease. Elevation of SGOT, SGPT ALP, bilirubin in blood often reflects hepatocellular damage. Resveratrol acts as a hepatoprotective agent is reported to have a protective effect on the plasma membrane of hepatocytes[17]. This study suggested that ability of resveratrol to condition the hepatocytes, accelerated regeneration of parenchyma cells thus protecting against membrane fragility and decrease of leakage of the marker enzymes into the circulation.

There is evidence that resveratrol has an intrinsic antioxidant capacity which depends on the redox properties of its hydroxyl phenolic groups and on the potential for the delocalization of electrons through the chemical structure. It has also been shown in some systems that this compound increases the activity of certain antioxidant and cytoprotective enzymes. The chemical induction of endogenous antioxidant and phase II enzymes varies among different types of tissues. In cardiomiocytes it has been shown that resveratrol increases the activities of various antioxidant and phase II enzymes which account for a marked cytoprotection against oxidative and electrophlilic injury[18].

Determination of plasma glutathione (GSH) may be valuable in the evaluation of liver disease, particularly in acute viral hepatitis and alcoholic liver disease in which the hepatic content of glutathione is suggested to be decreased. Such patients may be susceptible to oxidative stress and radical related hepatic injury. Glutathione, a tripeptide composed of glutamate, cysteine and glycine, which has multiple functions in disease prevention and in detoxification of chemicals and drugs while its depletion is associated with increased risk of toxicity and disease. GSH content in liver, blood and lymphocytes was significantly reduced in patients with hepatitis C. In present study we clearly explain that resveratrol treated group increased antioxidant, glutathione levels[19].

Histological findings clearly showed that the normal architecture of the hepatic tissue was altered due to administration of CCl_4 released reactive oxygen species causes degeneration in hepatocytes, extensive centrilobular necrosis around the central vein of liver. In contrast rats treated with resveratrol showed

noticeable improvement in histopathological parameters there was no sign of necrotic region, dilation of central vein. A part of literature suggested that resveratrol treatment is considered the most prominent on CCl_4 induced liver damage it protects primary rat hepatocytes against necrosis induced by reactive oxygen species [20].

In this study, we analyzed the inhibitory effect of resveratrol on the release of proinflammatory cytokines in CCl_4 induced rat hepatic damage model. Cytokines serves as central regulators controlling genes, responsible for both inducing apoptosis and enhancing cell damage or protection action on cell by stimulating proliferation of hepatocyte. Cytokines constitute a complex network involved in the regulation of inflammatory responses and maintain homeostasis of organ functions. Major hepatotoxic mediators in several experimental models of liver injury are TNFα and IL-6[21].

5. CONCLUSION

In present study the data first indicates that chronic CCl_4 treatment provoked a clear toxicity in rats, as assessed by biological alterations reflected by markedly increased and induced over expression of proinflammatory cytokines and we confirmed this toxic effect by histopathological studies showing that the CCl_4 damage to hepatic cells and the central vein. Protective effect of resveratrol was conformed in a well defined murine model of carbon tetrachloride induced liver injury. Recently much attention is focus on the protective functions of naturally occurring antioxidants in biological systems and their mechanisms. This study provides biological evidence that supports the use of resveratrol in the treatment of liver disorder. Thus, the present study reveals that resveratrol appears as a good candidate act as an antioxidant activity and a protective effect on CCl_4 induced hepatic damage.

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