Prevention of CCl_4 induced hypogonadism with *Raphanus sativus* seeds in rat

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Abstract: *Raphanus sativus* seeds are used as condiment and to treat hypogonadism, various ailments of liver and kidneys. The aim of this study was to evaluate the potential protective effects of methanol extract of *R. sativus* seeds (RSME) against hypogonadism induced with carbon tetrachloride (CCl_4) in Sprague-Dawley male rats. Thirty six rats were divided in to six groups with six animals in each. Animals of Group I were control and treated with saline, Group II, III and IV were given orally CCl_4 (1 ml/kg bw; 10% in corn oil). Rats of Group III and IV were also simultaneously given RSME at 100 mg/kg bw and 200 mg/kg bw respectively. However, Group V and VI received RSME (100; 200 mg/kg bw, respectively) alone. All treatments were given at alternate days for 15 days. Treatment of CCl_4 to rats decreased ($P < 0.001$) the level of CAT, POD, SOD, GST, GSH-Px and GSR antioxidant enzymes in testes of rat. Concentration of lipid peroxides (TBARS) was increased ($P < 0.001$) whereas concentration of GSH was decreased ($P < 0.001$) in testes of CCl_4 treated animals. Concentration of testosterone, FSH and LH in serum was decreased ($P < 0.001$) while the level of estradiol and prolactin was increased ($P < 0.001$) in CCl_4 treated rats. Injuries in seminiferous tubules were determined in histopathology of testes. Administration of RSME, dose dependently, markedly ameliorated the oxidative stress of CCl_4 thereby restoring the level of antioxidant enzymes, lipid peroxides, reduced glutathione, male hormones and alterations in histopathology.

Keywords: *Raphanus sativus* L. carbon tetrachloride, hypogonadism, antioxidant, lipid peroxidation.

INTRODUCTION

Male reproductive system is involved both in the exocrine (spermatogenesis) and endocrine (androgen synthesis) functions which require a precise regulation (Cheng and Mruk, 2009). Spermatogenesis is a continuous process occurs on the inner epithelium of seminiferous tubules in which spermatogonial stem cells mature in a stepwise way into specific germ cells before they are terminally differentiated to form spermatozoa (Shaughnessy *et al.*, 2009; Cheng and Mruk, 2010). Regulation of the whole spermatogenic process is dependent upon the hypothalamic-pituitary-testicular axis. Secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) is enhanced by the stimulation of gonadotropin releasing hormone (GnRH) of hypothalamus. FSH regulates the maturation of germ cells whereas LH binds to Leydig cells to stimulate testosterone synthesis (Pareek *et al.*, 2007; Shaha *et al.*, 2007).

Any minor disturbance either imposed internally or from the external environment such as chemicals, lead to fertility problems in males. Accumulation of reactive oxygen species (ROS) in testes induces hypogonadism that can be induced with carbon tetrachloride (CCl_4) exposure in experimental animals. CCl_4 is metabolized and bioactivated by cytochrome-P450 in to $\bullet CCl_3$ (trichloromethyl free radical) that reacts with macromolecules such as proteins, amino acids, fatty acid and nucleotides (Bruckner *et al.*, 2002). From $\bullet CCl_3$ even more reactive $\bullet CCl_3OO$ free radical is produced on reaction with oxygen. It initiates lipid peroxidation of unsaturated fatty acids by extracting hydrogen. Production of lipid peroxides induces destruction of membranes, protein inactivation and production of DNA reactive intermediates like carbonyls, aldehydes and lipid peroxy radicals (Halliwell and Gutteridge, 1999). Due to leakage of intracellular calcium storage compartments as a result of membrane breakage, calcium-dependent cellular endonuleases (that break DNA) and proteases that degrade proteins are activated (Halliwell and Guttering, 1999). Nucleases from lysosomes can also be released (Hashimoto *et al.*, 1995; Matsubara, 2002).These all nucleases can break and fragment DNA making condition more severe.

Excessive generation of ROS in testes disrupt its structure, function and signalling. Reactive oxygen species are produced as by products *in vivo* (Bloknina *et al.*, 2003). Normally cells maintain homeostasis of ROS at low levels through the action of intracellular enzymes and antioxidant metabolites. However, the prevalence of disease necessitates the consumption of antioxidants as therapeutic agents. Recent researches have established that plant extracts and /or their derived phytochemicals such as polyphenols, saponins, terpenoids, coumarins could have therapeutic importance in treating oxidative stress related diseases because of their free radical scavenging capacity (Ramchoun *et al.*, 2009).

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Raphanussativus L. (Brassicaceae) has been used as a food in many recipes particularly in salads, soups, sauces in Pakistan and many other countries. It is also used as medicine in eastern and western countries (Mayer, 1981). Its leaves are reported to enhance the protein digestibility, while roots are found effective in lowering the blood glucose level in diabetic rats (Chaturvediand Akala, 2001); restoration ability in hyper lipidemic rats (Yoshiaki, 2003). Aqueous and ethanol extracts of its sprout have been reported to exhibit hepatoprotective effect in rabbit (Zaman and Ahmad, 2004) while seed meal to enhance male rabbit fertility (El-Tohamy *et al*., 2010). Phytochemical investigation of radish seeds indicated the presence of alkaloid, coumarins, saponins, flavonoids and anthocyanins (Sanaa, 2001). Use of seeds decreases the uric acid level in the serum which is related to circulating markers of inflammation and free radical reactions (Zaman, 2004). Many physiological functions have been attributed due to anthocyanins in animal models. Anthocyanins comprised an important group of dietary antioxidants which protected the living cells from oxidative stress leading to prevention of diseases (Matsufuji *et al*., 2003). Moreover, isothiocyanates of radish seeds exhibited potential antimicrobial, antimutagenic and anticarcinogenic activities (Suh *et al*., 2006). Reports about using radish seeds against CCl_4-induced oxidative stress in testis are lacking. So, the present study aimed to monitor the effect of radish seeds crude methanol extract on the oxidative injuries induced with CCl_4 in Sprague-Dawley rats.

MATERIALS AND METHODS

Extract preparation

Fine powder of *R. sativus* seeds (1 kg) was extracted twice with 95% methanol and filtrate was dried under vacuum in rotator evaporator at 40°C yielding 6.3% of crude methanol extract of *R. sativus* seeds (RSME).

Animal treatment

Thirty six Sprague-Dawley male rats weighing 250±30 g were divided in to six groups. During the experiment the animals had free access to food materials. The animals were maintained in steel cages at the Primate Facility and the study procedure was approved by Ethical Committee of Quaid-i-Azam University, Islamabad. Animals of group I were treated orally with saline (0.5 ml/kg bw; 0.9% NaCl) whereas animals of group II, III and IV were administered orally with 1 ml/kg bw of CCl_4 (10% CCl_4 in corn oil). Rats of group III and IV also received RSME (100; 200 mg/kg bw, respectively) orally. A dose of 100 and 200 mg/kg bw was administered orally to animals of group V and VI alone. All animals received 8 doses in 15 days (alternate days). Animals of various groups were sacrificed after 24 h of the last treatment, blood was collected, centrifuged and serum was stored in refrigerator for further analysis.

Histopathological studies of testes

After sacrifice testes of all the animals were removed and immediately washed in ice cold saline. A portion of testis from each animal was processed for histology. Sections 4µmthickness were made when embedded tissues in paraffin. Hematoxylin/eosin stain was used for sections' staining and all slides were examined for various anomalies.

Estimation of antioxidant activity in testicular tissues

Second portion of the testis was fixed in liquid nitrogen and processed for the various biochemical studies. Supernatant obtained after homogenization of the tissues in phosphate buffer (100mM) containing EDTA (pH7.4; 1 mM) and centrifugation (12000 × g) at 4°C for 30 min, was used to estimate the protein contents (Lowery *et al*., 1951). Level of catalase (CAT) and peroxidase (POD) activity in various samples was determined by following the protocol of Chance and Maehly (1955). Spectrophotometric methods were used to determine the enzymatic activity of (SOD) superoxide dismutase (Kakkar *et al*., 1984); (GSR) glutathione reductase (Carlberg and Mennervik, 1975); (GST) glutathione-S-transferase (Habig *et al*., 1974); (GSH-Px) glutathione per oxidase (Mohandas *et al.,* 1984) in various samples. The quantity of lipid peroxides thiobarbituric acid reactive substances (TBARS) was estimated by applying the procedure of Iqbal *et al*. (1996) while the concentration of GSH in testes samples was estimated through the method of Jollow *et al*. (1974).

Estimation of hormonal level in serum

For quantitative estimation of hormones; testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol and prolactin in serum Micro LISA Quantitative EIA Test kits were used. Protocols given with kits were followed.

STATISTICAL ANALYSIS

Data obtained was represented as the mean and standard error (SE). The means of various treatments were compared for *post hoc* least significance difference (LSD) after one way analysis of variance by using SPSS version 14.

RESULTS

Effect of RSME on antioxidant enzymes

The activities of phase I antioxidant enzymes (fig 1A) and phase II antioxidant enzymes (fig 1B) in homogenates of testes of rats for different groups are depicted in fig1. Compared to control animals the activity level of CAT, POD and GSH-Px was decreased significantly ($P<0.001$) in Group II; the CCl_4 treated group. The toxicity of CCl_4 was ameliorated by the co-administration of RSME and a significant ($P<0.01$) increase in these indices was

recorded in Group IV; at maximum dose of 200 mg/kg bw in CCl_4 injured rats. However, the activity level of the above mentionedindices was non significantly (P 0.05) increased in Group III; RSME (100 mg/kg bw) along with CCl_4 treatment. Regarding the activity levels of SOD, GST and GSR, itwas found that these indices decreased significantly ($P < 0.001$) with CCl_4 treatment (Group II) as compared to the control samples. Activity level of SOD, GST and GSR was increased significantly at both the doses of RSME as compared to the CCl_4treated animals. The highest dose of RSME 200 mg/kg bw (Group IV) more markedly restored the level of these enzymes. However, the activity levels of all these parameters showed a non-significant change at both the doses of RSME alone (Group V and VI) as against the control samples.

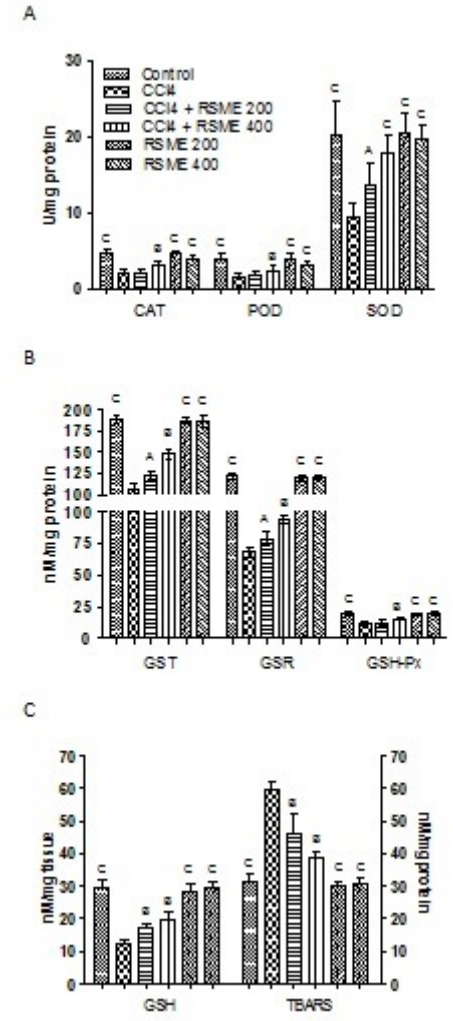

Fig 1: Protective effects of RSME on antioxidant enzymes in testis of rat. (A) Phase I antioxidant enzymes (B) phase II antioxidant enzymes (C) biomolecules of testes of rat. Mean ± SD (n=6 number). Significance from CCl_4 group A= $P < 0.05$, B= $P < 0.01$, C= $P < 0.001$

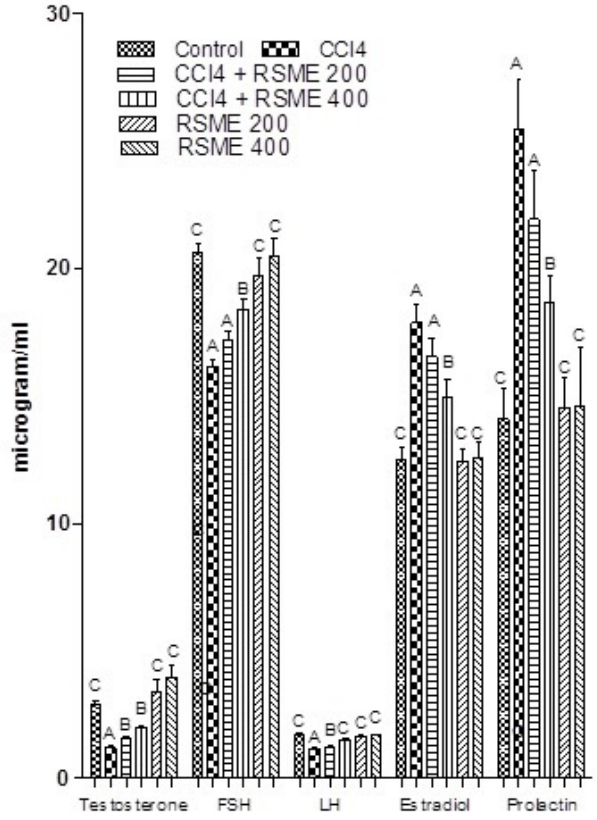

Fig 2: Protective effects of RSME on male hormones in rat. Mean±SD (n=6). Significance from CCl_4 group A= $P < 0.05$, B= $P < 0.01$, C= $P < 0.001$

Effect of RSME on reduced glutathione and lipid peroxidation

Toxicity of CCl_4 significantly ($P < 0.01$) decreased the concentration of reduced glutathione whereas increased ($P < 0.01$) the lipid peroxidation (TBARS) in the testis of rat compared with the control group (Figure 1C). Co-administration of RSME along with CCl_4 diminished the CCl_4-inducedchanges in the concentration of these parameters. These changes were more markedly restored towards the control group with 200 mg/kg bw dose in Group IV. However, non significant changes were recorded with RSME alone (Group V and VI) compared with the control samples.

Effect of RSME on serum reproductive hormones

Administration of $CCl_4$to rats (Group II) induced alterations in the concentration of male hormones such as testosterone, FSH, LH, estradiol and prolactin are shown in Figure 2. CCl_4treatment to rats (Group II) significantly ($P<0.001$) decreased the concentration of testosterone, FSH and LH whereas increased ($P<0.001$) the level of estradiol and prolactin comparatively to the control animals (Group I).Co-treatment of RSME at 100 mg/kg bw and 200 mg/kg bw showed significant protection ($P <0.01$) and reduced the toxic effects of CCl_4 dose

Table 1: Protective effects of RSME on testicular histology scores in rat

Treatments	Seminiferous tubules degeneration	Meiosis interruption	Sperm concentration	Germ cell morphology	Germinative epithelium
Control	-	-	-	-	-
CCl_4 (1 ml/kg bw)	++	++	++	++	++
RSME (100 mg/kg bw) + CCl_4	+	+	+	+	+
RSME (200 mg/kg bw) + CCl_4	+	-	-	+	+
RSME (100 mg/kg bw)	-	-	-	-	-
RSME (200 mg/kg bw)	-	-	-	-	-

-, normal; +, mild; ++, medium

dependently while restoring the altered level of testosterone, FSH, LH, estradiol and prolactin. Higher dose of RSME in Group IV more effectively restored the altered level of these hormones towards the control level. Treatment of RSME at both the doses 100 mg/kg bw (Group V) and 200mg/kg bw (Group VI) alone did not induce alteration ($P \wp 0.05$) in the level of these indices in serum of rats to that of the control animals.

Effect of RSME on histopathology of testis

Histopathological abnormalities were markedly visible with CCl_4 treatment in testis of rat (table 1). Administration of CCl_4 induced degeneration of seminiferous tubules, loss of germinativeepithelium, meiosis interruption along with abnormalities in germ cell morphology and reduction in sperm concentration within the seminiferous tubules. Co-treatment of RSME ameliorated the toxicity induced with CCl_4 and these abnormalities were diminished. At the lower dose of RSME100 mg/kg bw (Group III) mild form of injuries were still present while with the higher dose of RSME200 mg/kg bw(Group IV) markedly repaired and were comparable to normal histoarchitecture of testis.

DISCUSSION

Carbon tetrachloride (CCl_4) requires phase I cytochrome P4502E1 enzyme (CYP2EI) forbioactivation to form reactive metabolic CCl_3• (trichloromethyl radical) and •$OOCCl_3$ (peroxytrichloromethyl) radical with addition of oxygen (Weber *et al.,* 2003). These free radicals areable to bind with polyunsaturated fatty acid in cell membranes or endoplasmic reticulum to produce R• (alkoxy) and ROO• (peroxy radicals) that commence autocatalytic lipid peroxide molecules, leads to necrosis or degeneration of tissues (Hung, 2006; Shah and Khan, 2014). In this study CCl_4 treatment causes a significant decrease in activities of antioxidant enzymes; CAT, POD, SOD, GST, GSH-Px and GSR in testis constituted mutually interacting defense supportive team against ROS. Higher production of H_2O_2 inactivates SOD whereas GSH-Px and CAT protected SOD from the toxicity of H_2O_2.while SOD protected GSH-Px and CAT from superoxide anions. In this way they mutually act against free radicals generated in testis enforcing each other (Shagirtha *et al.,* 2011). There was also a decrease in GSH level and anincrease in the level of lipid peroxidation (TBARS) in the testicular tissues in this experiment. Since lipids are more easily vulnerable to attack by the CCl_4 metabolites. Peroxidation of polyunsaturated fatty acids produces TBARS that is a reactive aldehyde and its level shows the extent of tissue damage (Ohkawa *et al.,* 1979). Enhanced lipid peroxidation and inactivation of antioxidant enzymes may be responsible for the decrease in SOD level in testicular tissues. During CCl_4 toxicity decrease in the activity level of GSH-Px's could be due to the decrease inGSH contents as it is oxidized to GSSG. Effects of CCl_4 on antioxidant enzymes and lipid peroxidation in testis agree well with earlier reports (Khan and Ahmed, 2009; Khan *et al.,* 2011; Khan, 2012).

Administration of RSME magnified the activities of antioxidant enzymes of testis of CCl_4 treated rats. The level of lipid peroxidation was decreased however GSH level was enhanced in testis of RSME treated rats. These results showed that *R. sativus* seeds have antioxidant potential againstCCl_4 induced oxidative stress. These results were consistent with the data in literature that radish consumption reduces injuries induced by chemicals (Baek *et al*., 2008). Protection provided against oxidative stress by *R. sativus* extract was attributed to the presence of importantphy to chemicals most importantly coumarins, saponins, flavonoids, anthocyanins andisothiocyanates, acting as an antioxidant or they may induce detoxification of enzymes (Lee *et al.,* 2006; Shagirtha and Pari, 2011). *Raphanussativus* extract was also able to directly scavenge oxygen free radicals such as singlet oxygen, hydroxyl radical, hypochlorous acid, hydrogen peroxide and peroxyl radicals (Umamaheswari *et al.,* 2012).

Spermatogenic process works properly under the control of hypothalamopituitary-gonadal axis. Different hormones secreted by hypothalamus, pituitary and testes act together in an efficient way for the proper function and maintenance of reproductive system. Normal level of LH and testosterone is essential for maintaining normal function of antioxidant enzymes in testes (Palaniappan *et al.,* 2005). In this study, there was an oxidative stress induced decrease in the level of these hormones i.e., testosterone, FSH, LH in rats treated with CCl_4. Toxicity of CCl_4cause degeneration of Leydig's cells; the

testosterone producing cells. It resulted in decreased testosterone level (Khan and Ahmed, 2009). Leydig cells are more exposed to ROS because the yare in close proximity to testicular interstitial macrophages. So ROS induces lipid peroxidation in Leydig cell membrane leading to cell destruction and so reduced the testosterone production.

Reduced LH and FSH levels may be due to direct toxic effects of CCl_4 on pituitary i.e., central dysfunction (Khan and Ahmed, 2009). All these disruptions in hormones lead to hypogonadismin male rats. CCl_4 treatment also caused a rise in estradiol and prolactin hormones that are also characteristics of hypogonadism in males. Both hyperprolactinemia and hyperestrogenemiainduce feminization in males and may be involved in the central dysfunction-inducedhypogonadism in this study i.e., hypothalamic and pituitary dysfunction induced by CCl_4. All these toxic effects were ameliorated by administration of RSME in a dose dependent way.

Testosterone, LH, FSH levels were increased in CCl_4+RSME treated groups. At the same time prolactin and estrogen levels were decreased in CCl_4+RSME treated groups as compared to CCl_4treated group. RSME induce its curative effects by its antioxidant potential, scavenging free radical ability and may have important role in overcoming central-dysfunction by direct stimulating central nervous system and hypothalamus-pituitary-gonadal axis due to its phytochemicals.

Normal structure and function of testes is crucial for the normal spermatogenesis and steroidogenesis to take place. Both these processes contribute to the male fertility. Any change or

Disturbance in the structure and function of testes lead to hypogonadism in males. Testis tissue ishighly sensitive to toxic chemicals and reactive free radicals. Exposure to toxic chemicals leads to deleterious histological changes in testes. They cause degeneration of spermatogonial cells, so reduced or abnormal sperms' production (Khan, 2012). In our study CCl_4 administration induced

Partial degeneration of seminiferous tubules and germ cells proving spermiotoxicity (Khan and Ahmed, 2009). There was a decrease in number of spermatocytes in the tubules' lumen due to oxidative damage to these cells. Germinative epithelium thickness was also decreased in CCl_4treated rats. Meiotic process of spermatogonia was also interrupted. All these were restored when

RSME was co-administered with CCl_4. These results show that RSME has the potential of recovery towards normal morphology of testes. This beneficial effect of RSME may be due to stabilization of biological membranes due to its antioxidant potential and free radical scavenging ability.

CONCLUSION

Results of this study suggested that RSME constituted phytochemicals that ameliorates the oxidative stress in testicular tissues thereby a useful remedy for stress induced hormonal dysfunction in males. The high efficacy of this medicinal plant provides an alternative to conventional medicine.

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