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Cadmium-Induced Nephrotoxicity via Oxidative Stress in Male Wistar Rats and Capsaicin Protects Its Toxicity

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ABSTRACT

Cadmium (Cd) is one of the most toxic heavy metals used in various industries from where it leaks into the environment. It accumulates in the body, especially in the kidneys and damages the renal tubules which cause critical effects on health. Capsaicin, the active component of chili pepper which is the part of the diet, has anti-proliferative, anti-inflammatory and antioxidant properties. CdCl$_2$ was subcutaneously injected to rats; at 3 mg Cd/kg body weight, once a day for 8 days. Thereafter, from day 9 capsaicin 0.5 mg/kg/day was given to rats via i.p. for additional 7 days. On day 16, animals were sacrificed and kidneys were dissected out to analyze the biochemical parameters. Cd significantly increased the content of lipid peroxidation and decreased the level of glutathione and activities of antioxidant enzymes; glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase and capsaicin treatment significantly protected the contents of lipid peroxidation and glutathione and activities of antioxidant enzymes.The aim of the study was to treat the Cd toxicity with hot chili pepper constituent; capsaicin. The capsaicin is the part of the diet, those who are taking it routinely will be protected with mild toxicity of Cd. With this aim, we evaluated the mechanism of action of Cd and its protection with capsaicin.

Key words: Cadmium, capsaicin, lipid peroxidation, GSH, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase.

INTRODUCTION

Cd is one of the most toxic heavy metal used in industries of black polyethylene, black rubber, burned motor oil, cadmium alloys in dental prosthetics, ceramics, copper refineries, drinking water, electroplating, fungicides and nickel–cadmium batteries (1). It is also found in oysters and other sea foods, processed food, on the back of rubber carpet, rubber tires, sewage slug, silver polish, paint pigment, paper, pesticides, plastic tapes, galvanized pipes used for drinking water etc. (2). Fungicides sprayed on apple, tobacco and potatoes also contain Cd. Its exposure also disturbs the calcium metabolism, causing hypercalciuria and stones formation in the kidneys. High exposure can lead to lung cancer and prostate cancer. Cd is associated with diabetes, cardiovascular disease, and many other major degenerative diseases. Humans are exposed to Cd from time to time, because it is found in the air. Acute oral exposure to 20-30 mg of Cd caused fatalities in humans (3). Exposure to lower amounts may cause gastrointestinal irritation, vomiting, abdominal pain and diarrhea. Most studies have centered on the detection of early signs of kidneys dysfunction in the occupational setting (4).

Smoking cigarettes is very popular in Saudi Arabia and smoking is the biggest source of Cd toxicity, as it is found in tobacco. Cd is sprayed on the tobacco plant as a fungicide. It is also present in the cigarette papers. In each cigarette, the average residual cadmium concentration is 1.4 µg. Passive smokers also contain substantial amounts of Cd. One pack of cigarette deposits at least 4 µg into the lungs which is ten times the amount that the body can assimilate and excrete in a day. It retains in the kidneys, blood vessels, lungs and the brain. It weakens the immune system giving rise to the typical smokers' diseases: lung infections, lung cancer, emphysema, heart disease and malignant tumors.

Hot red chili pepper of genus *Capsicum,* is most heavily and frequently consumed spices throughout the world, have active ingredient phenolic substance capsaicin (8-methyl-*N*-vanillyl-6-nonenamide). It has

been used as a tool in the study of pain sensations which caused by stimulation of capsaicin receptor or vanilloid receptor-1, an ion channel protein expressed by nociceptive primary afferent neurons [5, 6]. It has analgesic and anti-inflammatory activities [7] and is currently used in topical creams and gels (e.g., Axsain and Zostrix) to mitigate neurogenic pain. Several studies examined its effects and observed protection on lipid peroxidation and nephrotoxicity induced by CDDP using various agents including antioxidants [8-10].

There are various routes through which humans are exposed especially the environment, but its treatment is limited. We explore the mechanism of action of Cd for the best treatment. The capsaicin is the part of the diet those who are taking it routinely will be protected with mild toxicity of Cd. With this aim, we evaluated the mechanism of action of Cd and its protection with capsaicin.

MATERIALS AND METHODS
CHEMICALS
Glutathione (oxidized and reduced), glutathione reductase, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), thiobarbituric acid (TBA), sodium azide, sulfosalicylic acid, 5-5′-dithio-bis-2-nitrobenzoicacid (DTNB), (−)-epinephrine, cadmium chloride, protease inhibitor cocktail and hydrogen peroxide were purchased from Sigma-Aldrich Co., Germany. Other chemicals were of analytical reagent grade.

ANIMALS
Male Wistar rats of weight 180-200 g were used in this study. The animals were procured from the Animal House of Jazan University, Jazan, Kingdom of Saudi Arabia and kept with 12 hours light dark cycle, light from 6:00 to 18:00 hr at temperature of 23 ± 2 °C. All animals were given free access to standard laboratory diet and tap water. All procedures were performed in accordance with NIH guidelines and the Guide for the Care and Use of Animals. Care and treatment of animals were approved and practices were performed according to approval of ethics regulation at the International Laws and Islamic Perspectives (11). These protocols were approved by the Jazan University, Institutional Animals Care and Use Committee (IACUC).

EXPERIMENTAL DESIGN
The rats were randomly divided into 4 groups, each having 8 animals. Group one served as controls and vehicle was given i.p. Group second served as experimental and $CdCl_2$ was injected subcutaneously at 3 mg Cd/kg body weight once a day for 8 days. The group third was treated with 3 mg Cd/kg for 5 days and thereafter animals were treated with capsaicin for 7 additional days, i.p. at a dose of 0.5 mg/kg. The fourth group was treated with capsaicin (0.5 mg/kg body wt). $CdCl_2$ was dissolved in saline solution and capsaicin in tween-80: ethanol: saline $(1:1:8, v/v)$. The dose of capsaicin and $CdCl_2$ was selected as described earlier (12, 13 respectively).

TISSUE PREPRATION
After 8 days of Cd treatment and additional 7 days of post treatment with capsaicin, the animals were sacrificed on day 16 by decapitation. Kidney was taken out, weigh and homogenized in 10 mM Tris-HCl, pH 7.4 having protease inhibitors cocktail (10 µL/mL) to give a 10% (w/v) homogenate and centrifuged at 1,000 g for 5 min at 4 °C to separate the nuclear debris. The supernatant-1 (S-1) was used for the assay of LPO and the remaining S-1 was again centrifuged at 12,500 g for 30 min at 4 °C to get post mitochondrial supernatant (PMS), which was used for other biochemical assays.

ASSAY OF LIPID PEROXIDATION
The procedure of Utley *et al.* (14) modified by Islam *et al.* (15) was used for the estimation of the rate of lipid peroxidation. In brief, S-1, 0.25 ml was pipetted in 15 x 100 mm test tubes and incubated at 37 ± 1 °C in a metabolic shaker (120 cycles/min) for 60 min. Another 0.25 ml of the same S-1 was pipetted in a centrifuge tube and placed at 0 °C. After 1 hr of incubation, 0.25 ml 5% chilled TCA followed by 0.5 ml of 0.67% TBA was added to each test tube and centrifuge tube and mixed after each addition. The aliquot from each test tube was transferred to centrifuge tube and centrifuged at 1,000 g for 10 min. Thereafter, supernatant was transferred to another tube and placed in the boiling water bath. After 10 min, the test tubes were cooled and the absorbance of the color was read at 535 nm. The TBARS content was calculated by using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nanomoles of TBARS formed/hr/mg of protein.

ASSAY OF GLUTATHIONE REDUCED
Reduced glutathione was assayed by the method of Jollow*et al*. (16). PMS 0.1 ml was precipitated with 0.1 ml sulfosalicylic acid (4%). The samples was kept at 4 °C for 1 hr, and then subjected to centrifugation at 1000 x g for 10 min at 4 °C. The assay mixture contained 0.1 ml aliquot, 0.8 ml phosphate buffer (0.1 M, pH 7.4) and 0.1 ml DTNB (0.4% in phosphate buffer 0.1 M, pH 7.4) in a total volume of 1.0 ml. The yellow

colour developed was read immediately at 412 nm. The GSH content was calculated as nmol GSH/ mg protein, using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

ASSAYS OF GLUTATHIONE PEROXIDASE

The method of Mohandas *et al*. (17) was used. In brief, the reaction mixture contained phosphate buffer (0.1 M, pH 7.0), EDTA (1.0 mM), sodium azide (1.0 mM), glutathione reductase (1.0 U/ml), glutathione (1.0 mM), NADPH (0.2 mM), hydrogen peroxide (0.25 mM) and 0.1 ml of PMS in a final volume of 1.0 ml. The disappearance of NADPH at 340 nm was recorded per min for a period of 3 min at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/mg/protein using molar extinction coefficient $6.22 \times 10^{-3} M^{-1} cm^{-1}$.

ASSAYS OF GLUTATHIONE REDUCTASE

Glutathione reductase activity was assayed by the method of Carlberg and Minerva (18) as modified by Mohandas *et al*. (17). In brief, the assay mixture contained phosphate buffer (0.1 M, pH 7.6), NADPH (0.1 mM), EDTA (0.5 mM), oxidized glutathione (1.0 mM) and 0.1 ml of PMS in a total volume of 1.0 ml. The enzyme activity was quantitated per min for a period of 3 min at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22 \times 10^{-3} M^{-1} cm^{-1}$.

ASSAY OF CATALASE

The activity of catalase was measured by the method of Claiborne (19). In brief, the assay mixture contained phosphate buffer (0.1 *M*, pH 7.4), hydrogen peroxide (6.0 mM) and 0.05 mL PMS to give a total volume of 1.0 mL. The change in absorbance per min for a period of 3 min was recorded at 240 nm. The catalase activity was calculated in terms of nmol of H_2O_2 consumed using molar extinction coefficient of 43.6 $M^{-1} cm^{-1}$.

ASSAY OF SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase activity was measured spectrophotometrically as described previously by Stevens et al. (20) by monitoring per min auto oxidation of (−)-epinephrine at pH 10.4 for 3 min at 480 nm. The reaction mixture contained glycine buffer (50 mM, pH, 10.4) and 0.2 ml of PMS. The reaction was initiated by the addition of (−)-epinephrine. The enzyme activity was calculated in terms of nmol (−)-epinephrine protected from oxidation/min/mg protein using molar extinction coefficient of 4.02×10^3 M^{-1} cm^{-1}.

ESTIMATION OF PROTEIN

Protein was estimated by the method of Lowry *et al.* (21) using bovine serum albumin as standard.

Statistics

The Students 't" test was used for the significance and p <0.05 was considered as significant.

RESULTS

EFFECT OF CADIMUM ON THE CONTENT OF LIPID PEROXIDATION AND GLUTATHIONE AND THEIR PROTECTION WITH CAPSAICIN

Fig. 1 shows the effect of Cd on lipid peroxidation and its protection in capsaicin treated animals. The thiobarbituric reactive substances were elevated significantly ($p < 0.001$) in Cd treated group as compared to control group. The treatment with 0.5 mg /kg b wt of capsaicin for 7 days has protected the content of TBARS significantly ($p < 0.001$) in Cd +Cp group as compared to Cd group.

The content of reduce glutathione (GSH) was decreased significantly ($p < 0.05$) in Cd treated group as compared to control and Cp has protected its content significantly ($p < 0.05$) in Cd+Cp group as compared to Cd group (Fig. 2).
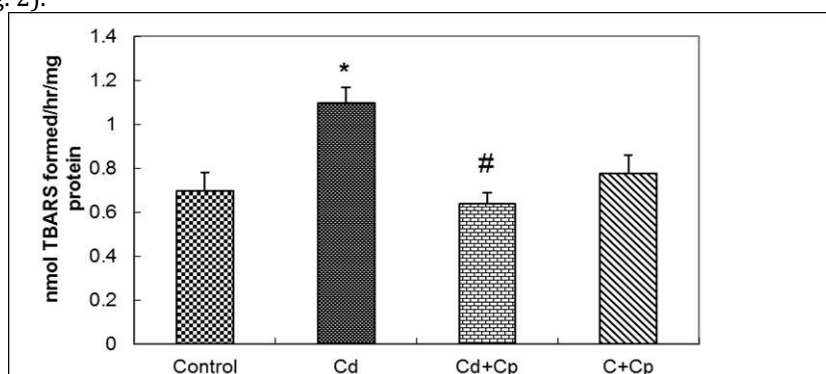

Fig 1.Effect of Cd on the content of TBARS and its protection by capsaicin. The elevated level of TBARS by Cd was protected significantly by Cp when compared with the Cd+Cp group. The values are expressed as mean ± S.E. (n=8). *$p < 0.05$ vs. control and #$p<0.05$ vs. Cd+Cp.
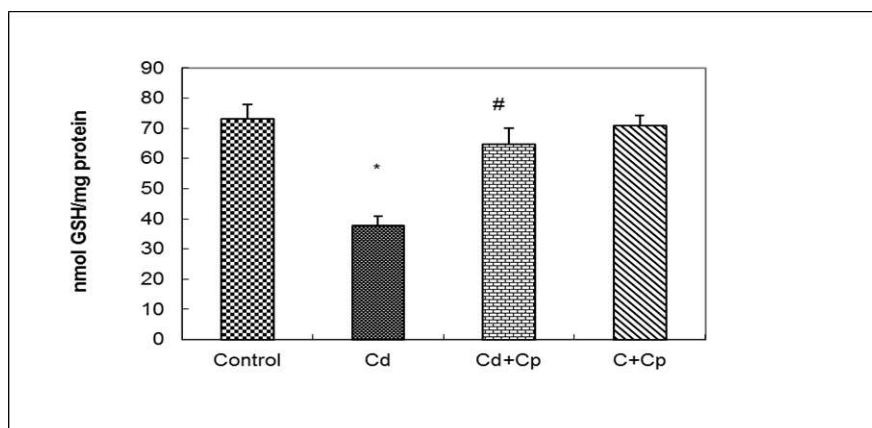
Fig.2. Effect of Cd on the content of GSH and its protection by capsaicin. The significantly depleted content of GSH in Cd treated group was protected significantly by Cp in Cd+Cp group. Values are expressed as mean ±S.E. of 8 animals. *p < 0.05 vs. control and #p < 0.05 vs. Cd+Cp.

EFFECT OF CADIMUM ON THE ACTIVITIES OF ANTIOXIDANT ENZYMES AND THEIR PROTECTION WITH CAPSAICIN

The activities of GPx and GR were depleted significantly (p < 0.01 and p < 0.001, respectively) in Cd treated group as compared to control group and it was protected significantly (p < 0.01 and p < 0.05 respectively) in the group treated with capsaicin (Cd+Cp) as compared to Cd treated group (Table-1). Similarly the activities of SOD and catalase were also depleted significantly (p < 0.01 and p < 0.001, respectively) in Cd treated group as compared to control group. The capsaicin has protected the activities significantly (p < 0.05 and p < 0.001, respectively) in Cd + Cp group as compared to Cd group (Table-1).

Table 1: Effect of cadmium on the activity of antioxidant enzymes and their protection with capsaicin

Enzyme	Control	Cd (3 mg/kg/day, s.c.) for 8 days	Cd+ Capsaicin (0.5 mg/kg/day, i.p.) for 7 days	Capsaicin (0.5 mg/kg/day, i.p.) for 7 days
GPx (nmol NADPH oxidized/min/mg/protein)	17.29 ± 0.66	13.44 ± 0.47** (-25.19 %) P < 0.01	15.39 ± 0.37## (11.42 %) P < 0.01	17.19 ± 0.46 (-0.58 %)
GR (nmol NADPH oxidized/min/mg/protein)	46.71 ± 2.01	19.75 ± 1.57*** (-57.72 %) P < 0.001	34.26 ± 2.31# (26.65 %) P < 0.05	47.23 ± 3.24 (1.11 %)
Catalase (nmol H_2O_2 Consumed/min/mg protein)	25.87 ± 0.32	5.13 ± 0.29*** (-80.14 %) P < 0.001	13.50 ± 0.46### (162.77 %) P < 0.001	26.14 ± 1.66 (1.04 %)
SOD (nmol epinephrine protected from oxidation/min/mg protein)	85.50 ±4.25	54.35 ± 4.15** (-40.04 %) P < 0.01	59.83 ± 1.95# (16.70 %) P < 0.05	85.11 ± 5.54 (-0.56 %)

Note: Values are expressed as Mean ± S.E.M. of 8 animals. Values in parentheses show the percentage change with respect to control.* p<0.05, ** p<0.01 *** p<0.001 vs control and # p<0.05, ## p<0.01 ### p<0.001 vs Cd.

DISCUSSION

Cd is a toxic heavy metal and its environmental contamination is recognized as a global problem. Cd is a highly accumulative toxicant with very long biological half-life (22). It is not biodegradable and its level in the environment is increasing due to industrial activities (22). Reactive free radicals such as superoxide and hydroxy can damage lipids, proteins and DNA and cause cell death (23). Cd is a potent inducer of oxidative stress and affects cellular antioxidant defense potential biophysically by inhibition and enhancement of several antioxidant enzymatic and non- enzymatic molecules.

Polyphenols have been reported to pose a membrane stabilizing activity by inhibiting the Cd induced generation of reactive oxygen species and maintain the structural integrity of the membrane (24). Lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an

important role in the toxicity of Cd (25). Cd induces oxidative stress by producing hydroxyl radicals, superoxide anions, nitric oxide and hydrogen peroxide (26, 27). The significantly increased level of lipid peroxidation has been reported in the kidney and various tissues treated with Cd in rats (28, 29). In the present investigation, Cd intoxicated rats has shown a significantly elevated level of lipid peroxidation which could be possibly due to excessive formation of free radicals which leads to the deterioration of biological macromolecules. Capsaicin treatment has shown a marked protection on the content of TBARS. This may be due to the presence of phenolic component, which has been recognized as an excellent scavenger of free radicals, thereby inhibiting lipid peroxidation and thus in agreement with the findings of others (8, 30).

Glutathione is an important antioxidant plays an important role in preventing the damage to cellular components caused by species such as free radicals and peroxides (31). It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the amine group of cysteine. A reduction in GSH might impair H_2O_2 clearance and promote $^\bullet$OH formation and, hence, oxidative stress. All antioxidant defenses are interrelated; the disturbance in one might derange the balance in all (32). The depletion in GSH content and enhancement of lipid peroxidation lead to the pathological changes in the kidney and consequently, lead to reduction in the activities of other antioxidant enzymes. The capsaicin has protected the contents of lipid peroxidation and GSH and antioxidant enzyme activities in the nephrotoxicity induced by capsaicin (30).

Superoxide dismutase (SOD) and catalase (CAT) constitute antioxidant defense against reactive oxygen species. SOD is a metalloenzyme that catalyzes the dismutation of superoxide radicals and converts superoxide into H_2O_2 (33, 34). In the present study the decreased activity of SOD in Cd treated rats was protected significantly with the treatment of capsaicin. Capsaicin has also been reported to protect the activity of SOD in the liver of Cd intoxicated rats (27). CAT is a hemeprotein which catalyses the reduction of H_2O_2 to water and oxygen and thus protects the cells from the oxidative damage of H_2O_2 and $^\bullet$OH (35). In the present study Cd has significantly decreased the activity of CAT which was protected significantly by capsaicin. Chance et al. (36) have reported a decreased activity of CAT in the liver intoxicated by Cd and its protection with capsaicin.

Kidney is an important organ of the body. Any change in its parameters may affect the functioning of the body as well as bad consequences of the health. So it is essential to evaluate the toxicity of Cd and its treatment. GPx is a seleno enzyme and plays a predominant role in removing excess free radicals and hydroperoxides and is a major defense system against oxidative stress. GR utilizes the NADPH and maintains the GSH in a reduced form (37). GSH is converted to GSSG by GPx, which is reconverted to GSH by GR, thus maintaining the pool of GSH, which in conjunction with the reluctant reduced NADPH, can reduce lipid peroxidase, free radicals and H_2O_2. The decreased activity of GPx and GR may be due to the decreased content of GSH in the kidney which was also supported by Moron et al. (37). The activities of GPx and GR were protected significantly by capsaicin in the nephrotoxicity induced by capsaicin (30). In the present investigation, Cd intoxicated rats showed a significant decreased activity of the antioxidant enzymes in kidney tissue which might be due to the over production of ROS, the primary mechanism of Cd toxicity (38).

CONCLUSION
Our results demonstrated that Cd increases the oxidative stress by depleting GSH and inhibiting the activities of antioxidant enzymes. The treatment with capsaicin significantly protected the Cd induced oxidative stress. The study suggests that capsaicin can be used as a cost effective safe herbal antioxidative agent in the treatment of Cd toxicity.

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DISCLOSURE STATEMENT
The authors declare that there are no competing interests.

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