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Original Research Article

Cardamom protects kidneys and decreases the expression of TRPM2 channels in doxorubicin- induced nephropathy

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Abstract: Doxorubicin (DXR) is a strong antineoplastic that has toxic side effects. In kidneys, DXR has been shown to have injurious effects. We investigated the protective effect of cardamom (CAR) on DXR- induced nephropathy (DIN). Four groups of six adult male Wistar Albino rats each were formed. During the 14- day experimental period, group I received no application, group II received 15 mg/ kg intraperitoneal (IP) dose of DXR, group III received 15 mg/ kg IP dose of DXR and 500 µl/ kg of CAR orally per day, and group IV received 500 µl/ kg of CAR orally per day. Transient receptor potential melastatin 2 (TRPM2) immunoreactivity, apoptosis by TUNEL staining, and malondialdehyde (MDA) levels were determined. The MDA levels of the DXR group were significantly higher than the control and CAR groups. The MDA levels of the DXR+ CAR group have significantly decreased in comparison with the DXR group. The prevalence of TRPM2 immunoreactivity was +1 in the control and CAR groups, +3 in the DXR group, and decreased to +1 in the DXR+ CAR group. Apoptosis in kidney tissue determined by TUNEL staining was similar in the control and CAR groups, but compared to the control group, there was a significant increase in the DXR group and and a significant decrease was observed in the DXR+ CAR group. We determined that CAR reduced MDA levels, apoptosis, TRPM2 expression and had potential antioxidant effects in DIN, and may be considered a preventative agent.
Keywords: Doxorubicin, apoptosis, nephropathy, TRPM2, cardamom

INTRODUCTION

Many drugs are metabolized and excreted from the kidneys. However, due to kidneys' exposure to the toxic effects of drugs and to a large amount of blood, their glomerular, tubular, and renal vascular structures may become damaged [1]. DXR is an anthracycline derivative antibiotic used in the treatment of many hematologic and solid cancers [2]. Although it is a broad spectrum and potent antineoplastic, its excess toxicity is a drawback [3]. DXR causes DIN, first by glomerular damage and then by tubulointerstitial injury. It causes changes to the glomerular filtration barrier— glycocalyx thickness decreases, the size of the pores of glomerular endothelial cells increases, glomerular charge selectivity decreases, and there is fusion of podocyte foot processes [4]. Several studies revealed that, reduction of antioxidant enzymes, increase of free radical formation and lipid peroxidation play major roles in the pathogenesis of DIN [5].

TRPM2 channels behave as Ca^{+2} channels or modify Ca^{+2} channels in the plasma membrane. Three factors are known to play roles in the opening of active TRPM2 cation channels— oxidative stress, ADP-ribose/ nicotinamide adenine dinucleotide metabolism, and tumor necrosis factor alpha. TRPM2 inhibition reduces oxidant stress and suggest that therapeutic approaches targeting TRPM2 may be effective in reducing renal I/ R injury [6].

Elettaria cardamomum (CAR) is a plant that grows in warm regions such as western and southern India and its seeds are also used as a spice [7]. Animal studies have shown that CAR has antioxidant effects, in addition to having antihypertensive, gastroprotective, spasmolytic, anti- bacterial, anti- platelet, and anti-cancer properties [8].

In this study, we aimed to investigate the effects of CAR on histopathological changes, TRPM2

channels, and oxidative stress on the kidney tissues of rats with DIN to evaluate whether CAR may be used as a therapeutic/ protective agent against DIN.

MATERIALS AND METHODS

Study protocol: Ethical approval for the study was obtained from local ethics committee. Eight-week old Wistar Albino male (250- 350g) rats were used. Rats were kept at a temperature of 21 ° C for 12 hours of light and 12 hours of darkness and their cages were cleaned daily. Baits were provided in steel containers, and water was available in glass bottles ad libitum. The rats were fed with rat chow.

Four experimental groups were established as 6 animals each group, according to the ethical instructions: The control group (n = 6): There were no transactions during the 14- day study. DXR group (n = 6): A single dose of 15 mg/ kg IP DXR- hydrochloride (C27H29NO11HCl) (Doxo Teva 50 mg vial) was administered on the first day of the experiment. DXR+ CAR group (n = 6): A single dose of 10 mg/ kg IP DXR- hydrochloride (C27H29NO11HCl) (Doxo Teva 50 mg vial) was administered on the first day of the study and, 462 mg/kg/day CAR (Sigma Aldrich W224111 USA) was administered orally each day during the experiment. CAR group (n = 6): Throughout the experiment 462 mg/kg/day CAR was administered orally each day. In the end of the experiment, all rats were administered ketamine (75 mg/ kg) + xylazine (10 mg/ kg) under an IP anesthesia procedure and then decapitated. The kidney tissues were removed quickly after decapitation. A portion of the removed kidney tissues was fixed in 10% formalin solution for histological studies. For MDA and PCR analysis, other sections of tissues were stored at - 80 ° C. Doses of doxorubicin and cardamom, and the time of the experiment were performed according to previous studies in the literature.

Determination of MDA levels

For MDA analysis, a buffer solution was prepared with 0.42 g Tris-base + 1.43 Tris-HCl +3 g KCl, and 0.5 ml Tween 20 in 250 ml distilled water. This buffer was used to homogenize the samples. Next, 5 ml of the buffer were added to 1 g of tissue, and the tissue was completely disrupted with the homogenizer (Ultra-Turax T25, IKA-Labortecknik). The homogenate was centrifuged at 5000 rpm for 5 minutes, and 1 ml of the supernatant fraction was transferred to a new tube. After that, 1 ml of 10% Tri-chloro acetic acid (TCA), 1 of ml 0,067 % 2-thio barbituric acid (TBA), 1 ml of distilled water, and 0.5 ml of 4% HCl were added to 1 ml of the sample. The prepared mixture incubated at 90ºC for 120 minutes. After incubation, the tubes were cooled at room temperature, and the mixture was vortexed after the addition of 3 ml butanol. The tubes were centrifuged at 5000 rpm for 5 minutes, and the resulting supernatant was analyzed using spectrophotometry against butanol at 532 nm.

TUNEL method

For TUNEL method, sections 4-6 µm in thickness obtained from paraffin blocks were applied to polylysine slides. Apoptotic cells were identified using Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kits (Chemicon, catno: S7101, USA) in accordance with the manufacturer's instructions. Preparations were reviewed, evaluated, and photographed with the research microscope (Olympus BH-2). In the evaluation of TUNEL staining, nuclei stained with Harris hematoxylin blue were normal, while brown nuclear staining was interpreted as representing apoptotic cells. At 10 times magnification in randomly selected areas, at least 500 normal and apoptotic cells were counted. The proportion of apoptotic cells compared with the total (normal/ apoptotic) number of cells was calculated as the apoptotic index (AI). The scale bar was 50µm.

Immunohistochemistry Method

For immunohistochemistry analysis in order to determine TRPM2 channels, sections 4-6 µm in thickness taken from paraffin blocks were collected on polylysined slides. Deparaffinized tissues were passed through a graded alcohol series and boiled in a citrate buffer at a pH of 6 in a microwave oven (750 W) for 7 + 5 minutes. The tissues were cooled for about 20 minutes at room temperature after boilin and then were washed with 0.01M Phosphate Buffered Saline (PBS, P4417, Sigma-Aldrich, USA) for 3x5 minutes. The tissues were incubated in hydrogen peroxide (Hydrogen peroxide Block, TA-125-HP, LabVision Corporation, USA) for 5 minutes to to block endogenous proxidase activity. After washing with PBS for 3x5 minutes, Ultra V Block (TA-125-UB, LabVision Corporation, USA) was administered for 5 minutes in order to prevent background painting. Tissues were incubated in 1/200 diluted primary polyclonal antibody (Rabbit Anti-TRPM2 antibody, ab101738, Abcam, Cambridge, UK) in a humid environment for 60 minutes at room temperature. Tissues were washed with PBS for 3x5 minutes after the application of the primary antibody and incubated with the secondary antibody (biotinylated Goat Anti-Poliyvalent (anti-mouse/ rabbit IgG), TP–125-BN, Lab Vision Corporation, USA) in a humid environment for 30 minutes at room temperature. Tissues were washed with PBS for 3x5 minutes after application of the secondary antibody and incubated with Streptavidin Alkaline Phosphatase (TS-060-AP, Lab Vision Corporation, USA) in a humid environment for 30 minutes at room temperature. Fast Red Substrate System (TA-125-AF, Lab Vision Corporation, USA) was added drop wise to the tissue after the video signal

was received by the light microscope. Counterstaining textures of tissues were created using Mayer's hematoxylin staining then rinsed to PBS and distilled water closed with proper closing solution (Large Volume Vision Mount, TR-125-UG, LabVision Corporation, USA). Preparations were examined, evaluated and photographed with an Olympus BX 50 microscope. Normal rabbit IgG was used for negative control group. The immunohistochemical histo-score was created on the basis of immunoreactivity prevalence (0.1: <25%, 0.4: 26-50%, 0.6: 51-75%, 0.9: 76-100%) and severity (0: no, +0.5: very little, +1: little, +2: medium, +3: severe). Histo-score = prevalence × severity.

Statistical analysis

Data were analyzed using statistics software package (SPSS® for Windows, version 12.0). Results were presented as mean± standard deviation. Statistical significance of the data obtained with student T "paired" and one-way ANOVA tests. *p* values lower than 0.05 were regarded as statistically significant.

RESULTS

MDA Levels: MDA levels, which were measured spectrophotometrically, were similar in the control and CAR groups. Compared to the control and CAR groups, the MDA levels in the DXR group were significantly increased ($p < 0.05$). On the other hand, the MDA levels in the kidney tissue of the DXR+ CAR group were significantly decreased in comparison with DXR group ($p < 0.05$) (Table 1).

Immunohistochemical Findings- TRPM2 Immunoreactivity: The immunohistochemistry results for TRPM2 immunoreactivity, which were examined under light microscopy revealed that TRPM2 immunoreactivity in kidney tissue was observed only in the tubules. The prevalence of TRPM2 immunoreactivity was +1 in the control group. TRPM2 immunoreactivity of the DXR group was significantly increased in comparison with the control group with +3 prevalence. TRPM2 immunoreactivity was +1 in the DXR+ CAR and the CAR groups (Figure 1a).

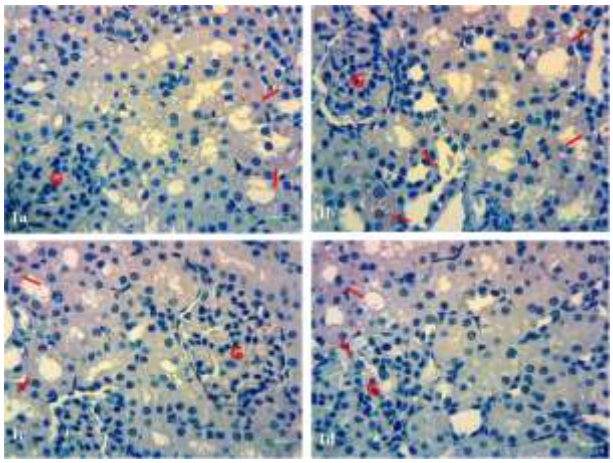

Fig-1a: Prevalence of TRPM2 immunoreactivity in the kidney tissues of the groups (Scale bar: 50 µm); 1a: Control, 1b: DXR, 1c: DXR+ CAR , 1d: CAR, →: immunoreactivity, G: glomerulus.

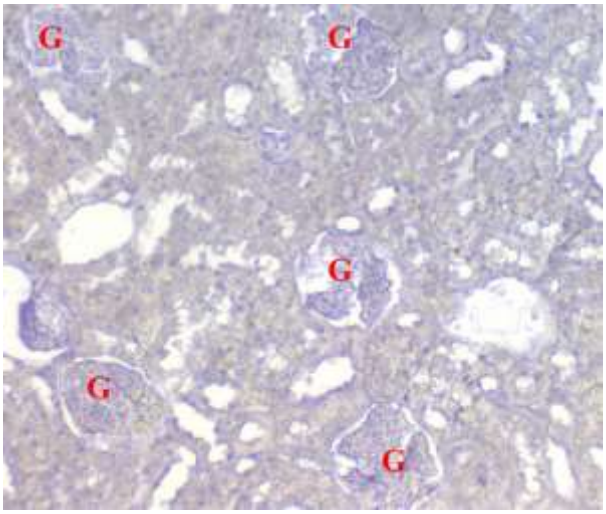

Fig-1b: The immunohistochemistry labeling in the kidney tissue of a negative control rat (G: glomerulus).

TUNEL Findings: The results of TUNEL staining, done to determine apoptotic cells under light microscopy, revealed that TUNEL positivity in kidney tissue was similar in the control and CAR groups. The DXR group had increased staining in comparison with the control group ($p < 0.05$), and the DXR+ CAR group had less staining than the DXR group ($p<0.05$) (Figure 2) (Table 1).

Table-1: Apoptotic index, MDA levels and TRPM2 immunoreactivity of groups

	Apoptotic index (%)- TUNEL	MDA levels (nmol/ mL)	TRPM2 immunoreactivity/ histoscore
Control	3,17±1,17	132,65±4,42	0.34±0.11
DXR	16,17±2,79[a]	252,74±3,06[a]	2.40±0.46[a]
DXR+ CAR	7,83±0,98[b]	200,36±45,22[b]	0.38±0.03[b]
CAR	2,83±0,41	119,88±11,64	0.31±0.11

Values are expressed as mean±standard deviation, [a] in comparison with the control group, [b] in comparison with the DXR group ($p < 0.05$).

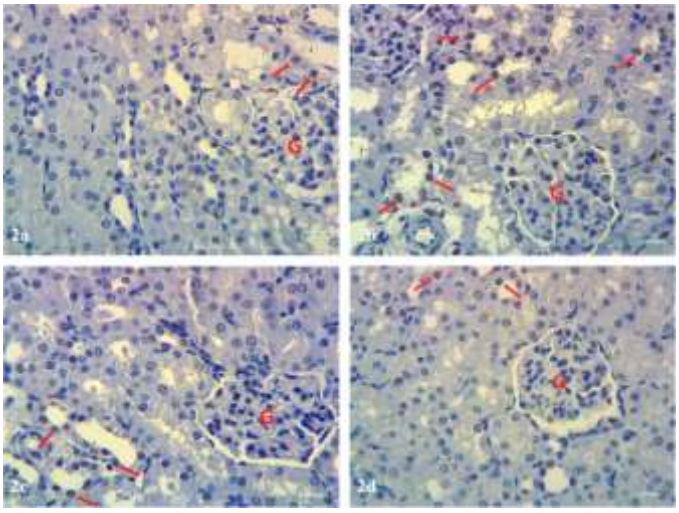

Fig-2: TUNEL positive cells in the kidney tissues of the groups (Scale bar: 50 µm); 2a: Control, 2b: DXR, 2c: DXR+ CAR , 2d: CAR, →:TUNEL positive cells, G: glomerulus

DISCUSSION

Most chemotherapeutic drugs are metabolized and excreted through the kidneys. Drugs and their metabolites can cause kidney damage, ranging from non-symptomatic creatinine elevation to renal failure, which requires dialysis [4].

DXR is an effective antineoplastic drug used in treatment protocols for Hodgkin's lymphoma, Non-Hodgkin's lymphoma, acute lymphoblastic leukemia, metastatic breast cancer, ovarian cancer, lung cancer, and sarcomas. Even though it is advantageous in its broad spectrum applicability and potency, it also has various toxic effects.[2] DXR may cause nephrotoxicity in experimental models and in humans [9,10].

Oxidative damage can occur in all biological molecules, including DNA, lipids, proteins, and carbohydrates. All biological structures, particularly lipids, may be damaged by attack by free radicals, and DXR administration is one cause of imbalance between free radicals and antioxidants. The confusion in the oxidant and antioxidant systems results in tissue damage, which occurs through tissue protein oxidation and lipid peroxidation. Membrane damage caused by lipid oxidation is irreversible but can be terminated by antioxidant reactions because antioxidants suppress lipid peroxidation by preventing peroxidation chain reactions and/ or by collecting the reactive oxygen species [11,12].

Some studies with experimental animal models of DIN have determined that as oxidative stress increases, enzymatic and/ or non- enzymatic antioxidant defense systems decline. In the study of Kalaiselvi *et al*. [13], after application of 10 mg/ kg intravenous DXR, enzymatic antioxidant levels (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and glutathione-S-transferase) were found to decrease while MDA levels increased. After identifying the role of free radicals and antioxidant enzymes in the pathogenesis of DIN, further studies have run antioxidant therapy trials. In many such studies, antioxidants were shown to significantly decrease kidney damage in DIN. For example, in our previous study about the effects of vitamin D on DIN,

we observed its protective effects on the kidney tissues of rats treated with DXR [14].

Various antioxidants are used to reduce oxidative stress and to increase antioxidant synthesis, including many herbal antioxidants. Similarly, many studies on the effects of antioxidants against DIN have been conducted with positive results. CAR is one such herbal antioxidant with many positive anti-inflammatory and anti- oxidant effects [15]. Multiple experimental studies have shown CAR to have antioxidant, antihypertensive, gastroprotective, spasmolytic, antibacterial, antiplatelet, and anti-cancer properties. In addition, Nair *et al*. [16] showed that CAR contains antioxidant phenolic and flavonoid compounds.

In this study, as in many experimental models, successful nephrotoxicity was induced in rats that were administered DXR. The MDA levels of the DXR group significantly increased in comparison with the control and CAR groups ($p < 0.05$) and the DXR+ CAR group had significantly decreased MDA levels in kidney tissue compared to the DXR group ($p < 0.05$). DXR causes significant lipid peroxidation in heart, kidneys, and liver, and one of the most important indicators of this process is increased levels of MDA [17]. The data obtained in our study were consistent with the literature and all these findings support the possibility of protective effects of CAR against DIN by interfering with antioxidant systems.

Ion channels also play a very important role in cellular membranes for the processes necessary for maintaining cell viability and cellular functions [18]. TRPM2 channels have been demonstrated in many tissues and cells, including the brain, bone marrow, kidney, intestine, liver, lung, testis, prostate, pancreas, skeletal muscle, leukocytes, and dorsal root ganglia [19]. TRPM2 channels behave as Ca^{+2} channels or modify Ca^{+2} channels in the plasma membrane [6]. Three factors are known to play roles in the opening of active TRPM2 cation channels— oxidative stress, ADP- ribose/ nicotinamide adenine dinucleotide metabolism, and tumor necrosis factor alpha [20]. As previously mentioned, TRPM2 channels are one of the ion channels in the cell membrane that are activated by oxidative stress [21]. Similarly, Hara *et al*. [20] and Wehage *et al*. [22] demonstrated in their experimental studies that TRPM2 channels are activated by oxidative stress. And also TRPM2 channels are shown to be physiologically important in oxidative stress- induced cell death [23].

Furthermore, Gao *et al*. [24] evaluated the nonselective cation channel TRPM2 in an experimental kidney ischemia/ reperfusion (I/ R) injury model. TRPM2- deficient mice were resistant to ischemic injury and had better kidney function, less histologic damage, suppressed proapoptotic pathways, and reduced inflammation. Also, pharmacologic TRPM2 inhibition was protective against I/ R injury. TRPM2 was localized mainly in kidney proximal tubule epithelial cells, and studies indicated that the effects of TRPM2 are mainly due to its expression in parenchymal cells. These results demonstrate that TRPM2 inhibition reduces oxidant stress and suggest that therapeutic approaches targeting TRPM2 may be effective in reducing renal I/ R injury.

In our study, TRPM2 immunoreactivity was only observed in kidney tubules. A significant increase in TRPM2 immunoreactivity was observed in the DXR group in comparison with the control group. Furthermore, treatment with CAR in the DXR+ CAR group induced a significant decrease in TRPM2 immunoreactivity.

The limitations of this study are; relatively small sample size and impossibility of TRPM2 measurement by immunoblotting method.

CONCLUSION

Thus, this study revealed that CAR reduced apoptosis; reduced the expression of TRPM2 ion channels, which are considered an oxidation source; reduced MDA levels, which indicates lipid peroxidation and has protective effects on renal tissue, probably due to its antioxidant properties. In the light of these findings, we believe that CAR can be used as an alternative treatment option to reduce the toxic effects of chemotherapeutics such as DXR in the future.

Declaration of Conflicting Interests: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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