Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2016-0450.R1
Manuscript Type:	Article
Date Submitted by the Author:	01-Dec-2016
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Keyword:	Cyclophosphamide, carvacrol, nephrotoxicity, oxidative stress, cytoprotection

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Carvacrol attenuates cyclophosphamide-induced oxidative stress in rat kidney

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ABSTRACT

Cyclophosphamide (CP) is an antineoplastic drug inducing kidney damage via producing oxidative stress. Carvacrol (CAR) has antioxidative effect and we postulated that it can be protective against CP-induced nephrotoxicity. Six groups (n=7) of rats (control, 100 mg/kg CP, CP+5 and CP+10 mg/kg CAR and 5 and 10 mg/kg CAR) were injected intraperitoneally. Serum malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), creatinine (CRE), total antioxidant capacity (TAC) and total oxidant state (TOS) were measured, and oxidative stress indexes (OSI) were calculated. Kidneys were also analyzed histologically. In CP alone group MDA, CRE, TOS and OSI levels increased whereas GSH, SOD, CAT, and TAC levels decreased compared with control group. In CP plus CAR groups MDA, TOS and OSI levels decreased whereas GSH, SOD, CAT and TAC levels increased compared with CP alone group. However, CRE levels were similar in CP alone and CP+5 CAR group whereas decreased in CP+10 CAR group. CP+10 CAR group was significantly different in all parameters (except TAC) from CP+5 CAR group. Kidney microscopy was showed lower tissue damage in CP plus CAR groups. In conclusion, 10 mg/kg CAR is more effective than 5 mg/kg CAR in prevention of CP-induced oxidative damage on kidney.

Keywords: Cyclophosphamide, carvacrol, nephrotoxicity, oxidative stress, cytoprotection.

INTRODUCTION

One of the most important and widely used drugs is cyclophosphamide (CP). CP is an alkylating antineoplastic agent that damages normal cells while killing cancerous cells in vivo during chemotherapy (Jurado-Garcia et al. 2008; Olayinka et al. 2015). The major restriction of CP therapy is the injury of normal tissue, leading to multiple organ toxicity mainly in bone marrow, testes, urinary bladder and kidney (Ahmed et al. 2015; Liu et al. 2012; Saba et al. 2013). CP's active metabolite acrolein intervenes in the tissue antioxidant defense system and produces highly reactive oxygen-free radicals (Ceribasi et al. 2010; Kern and Kehrer 2002; Mythili et al. 2004). In order to prevent these toxic side effects, antioxidants should be used to detoxify the acrolein during CP chemotherapy. Thus, there is a need for a novel agent that should protect the normal tissue from CP induced toxicity.

Recent studies showed that reactive oxygen species play an important role in CP-induced nephrotoxicity. For example, one of the indicators of oxidative stress, namely malondialdehyde (MDA) increases in kidneys after CP administration, but levels of important cellular antioxidant namely superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) decrease (Abraham and Rabi 2011; Adatiya and Jaisawal 2015; Said et al. 2016).

Various parameters have been used widely to measure total oxidant status (TOS), total antioxidant capacity (TAC) and oxidant stress index (OSI) as safe markers (Erel 2005).

Carvacrol (CAR) is a major natural constituent of essential oil fraction of aromatic plants such as oregano and thyme. It is a monoterpenic phenol biosynthesized from γ -terpinene, through p-cymene. It has been indicated that CAR has strong antioxidant effects at different doses in many studies (Suntres et al. 2015). In addition, CAR is reported to exhibit fungicidal (Ahmad et al. 2011), insecticidal (Tang et al. 2011) and antimicrobial activities (Nostro and Papalia

2012). We aimed in this study to investigate the potential protective effect of CAR using two different doses against CP-induced oxidative stress and nephrotoxicity in rats.

MATERIALS AND METHODS

Experimental Protocol

Forty-two male *Sprague-Dawley* rats, weighing 200 ± 20 g, were supplied by Experimental Animal Research Facility of Eskisehir Osmangazi University, Turkey. All experimental procedures were conducted in accordance with the guidelines of Eskisehir Osmangazi University Institutional Animal Ethics Committee (Protocol no: 213/2011) and with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences (Institute for Laboratory Animal Resources 1996).

Animals were housed under standard conditions of humidity (50%), temperature (25 ± 2 °C) and a 12-h light/12-h dark cycle. They were fed with pellets and tap water *ad libitum*, and divided in a random manner into 6 groups, seven in each. Animal groups and their drug administrations are summarized in Table 1. We used CP at a dose of 100 mg/kg (Souza-Filho et al. 1997), CAR at 5 or 10 mg/kg and corresponding saline intraperitoneally (Ayhanci et al. 2011; Azirak and Rencuzogullari 2008).

Blood Sample Collection

Twenty-four hours after the last saline or doses of CAR injections, rats were anesthetized with an i.p. injection of ketamine (50 mg/kg)/xylazine (10 mg/kg) and then sacrificed. Blood samples were collected from retro-orbital venous plexus into non-heparinized tubes. Serum was separated by centrifugation of blood samples at $4000 \times g$ for 20 min and stored at -20°C .

Histological Procedures

Kidneys were quickly removed, washed in cold saline and then cut into pieces of about 1 cm³. Tissues were fixed in 10% buffered formaldehyde, dehydrated in graded ethanol series, cleared in xylol and embedded in paraffin (Sigma-Aldrich Company, St. Louis, MO, USA). Four to 5 µm thick serial sections were cut, stained with hematoxylin-eosin (Sigma-Aldrich) and then examined by light microscopy in terms of hemorrhage, inflammation, necrosis, edema, tubular degeneration, glomerular degeneration, and narrowing of Bowman's capsule space.

Biochemical studies

Malondialdehyde (MDA) assay (TBARS; Thiobarbituric Acid Reactive Substances, TBA-MDA). Lipid peroxidation in freshly thawed serum was assessed by measuring MDA formation, using the TBA assay (Yagi 1994). The principle of the method depends on the measurement of pink color produced by the interaction of TBA with MDA. About 0.3 mL of serum was mixed with 2.4 mL of 0.042 M H₂SO₄ and 0.3 mL of 10% phosphotungstic acid (Sigma-Aldrich). After incubation at room temperature for 5 min, the mixture was centrifuged at 1600 ×g for 10 min, supernatant discarded and then pellet resuspended in 4 mL of distilled water. Subsequently, 1 mL of 0.67 % TBA (Sigma-Aldrich) was added and the mixture was heated in boiling water for 60 min. It was then centrifuged again at 1600 ×g for 10 min and absorbance of the organic layer was read spectrophotometrically at 532 nm. Tetramethoxypropane (Sigma-Aldrich) was used as a standard and MDA levels were calculated as nmol/mL. All analyses were repeated at least three times.

Glutathione (GSH) assay. Levels of reduced GSH were determined by colorimetric assay kit (Cayman Chemical Company, Canada) according to the manufacturer's protocols.

Absorbance of samples was measured at 405 nm using VERSA max tunable microplate reader (Molecular Devices, California, USA) and the results were expressed as μM .

Superoxide dismutase (SOD) activity assay. Levels of SOD were determined by colorimetric assay kit (Cayman) according to the manufacturer's protocols. Absorbance of samples was measured at 450 nm using VERSA max tunable microplate reader (Molecular Devices). Results were given as U/mL.

Catalase (CAT) activity assay. According to the manufacturer's protocols, levels of CAT were determined by colorimetric assay kit (Cayman). Absorbance of samples was measured at 540 nm using VERSA max tunable microplate reader (Molecular Devices). Results were indicated as nmol/min/mL.

Measurement of creatinine (CRE) levels. Levels of CRE were also determined by colorimetric assay kit (Cayman) according to the manufacturer's protocols. Absorbance of samples was measured at 490 nm using VERSA max tunable microplate reader (Molecular Devices) and the results were expressed as mg/dL CRE concentration.

Measurement of total antioxidant capacity (TAC) and total oxidant status (TOS). Serum TAC levels were measured in mmol Trolox equivalent/L using method developed by Erel (2004). Serum TOS levels were calculated in $\mu\text{mol H}_2\text{O}_2$ equivalent/L according to the method as previously described by Erel (2005).

Calculation of oxidative stress index (OSI). The ratio of the total peroxide to the total antioxidant potential gave the OSI, an indicator of the degree of oxidative stress (Aycicek et

al. 2005). OSI (Arbitrary Unit; AU) = TOS ($\mu\text{mol H}_2\text{O}_2$ equivalent/L) / TAC ($\mu\text{mol Trolox}$ equivalent/L) $\times 100$.

Statistical Analyses

Data obtained from each experiment were expressed as mean $\pm$ SD and $p < 0.05$ was accepted as statistically significant. The significance of the difference between the parameters of all groups were assessed using one-way analysis of variance for CRE, MDA, GSH, SOD, CAT, TAC, TOS and OSI levels. The Tukey's test was then performed to analyze the two groups consecutively. All statistical analyses were carried out using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Histology

Histological evaluation of kidneys of saline and CAR alone treated animals showed a normal structural appearance (Figure 1). CP alone treated rats showed inflammation foci, edema, glomerular atrophy, desquamation or vacuolization in tubular epithelial cells and narrowing in Bowman capsular space in kidney tissue. Addition of 10 mg/kg CAR provided a marked protection but 5 mg/kg CAR demonstrated a partial amelioration in CP-induced kidney damage (Figure 2).

Biochemical assays

Comparisons of the serum levels of CRE, MDA, GSH, SOD, CAT, TAC, TOS and OSI between the groups are shown in Figure 3-10. In CP alone treated group MDA, CRE, TOS, and OSI increased ($p < 0.05$), but GSH, SOD, CAT and TAC decreased ($p < 0.05$) compared to

the control group. When compared with CP alone treated group, administration of respective doses of CAR (5 and 10 mg/kg) together with 100 mg/kg CP ameliorated CP-induced oxidative stress, as indicated by decreases ($p<0.05$) in MDA, CRE (only with 10 CAR), TOS and OSI, and by increases ($p<0.05$) in GSH, SOD, CAT and TAC. When we compared CP+5 mg/kg CAR with CP+10 mg/kg CAR, we determined that 10 mg/kg CAR is more effective ($p<0.05$) than 5 mg/kg CAR according to all the parameters except TAC.

DISCUSSION

CP-induced renal damage has usually been neglected since plasma CRE, an indicator of glomerular function of the kidney, is not altered conspicuously in patients on CP chemotherapy (Abraham and Rabi 2011; Estakhri et al. 2013). It has been reported that CP causes renal damage at histological level in rats despite of unaltered plasma CRE levels (Sugumar et al. 2007). In the current study, we observed that CP administration increased serum CRE level significantly (Figure 7) and caused histopathological alterations in renal tissue (Figure 2). Similar findings were also reported by Sayed-Ahmed (2010), demonstrating that CP-induced acute renal damage was detected by the increases in serum CRE and blood urea nitrogen, and also with the presence of moderate histopathological lesions in kidney tissues.

CP toxicity consists of the cumulative effect of two mechanisms: Firstly, the levels of nucleophiles such as GSH decrease by interaction with acrolein and secondly, the peroxynitrite is produced by coupling of O_2^- and nitric oxide (Bhatia et al. 2008). GSH exerts its antioxidant function by reacting with superoxide radicals, peroxy radicals and singlet oxygen followed by the formation of oxidized GSH and other disulfides (Meister 1998). MDA is the end product of lipid peroxidation, which is a reactive aldehyde and marker of

oxidative stress (Kim et al. 2012). In the present study, treatment of rats with CP resulted in GSH depletion and increased MDA levels (Figure 3 and 4).

Tissue damage occurs when the cellular antioxidants are depleted. CP can induce changes in endogenous antioxidant system including CAT or glutathione peroxidase and GSH levels together with SOD, one of the most powerful antioxidant in the cells (Ettaya et al. 2016; Korkmaz et al. 2007). In our study, we determined significant decreases in SOD and CAT activities (Figure 5 and 6) in CP alone treated rats. It is possible that this decrease is a result of modification of protein structure through the reactive metabolite acrolein and/or reactive oxygen species generated during CP metabolism.

TOS and TAC are reliable in terms of giving the body's net stress. In this study, we used TAC and TOS, a novel technique developed by Erel (2004) in the full determination of oxidant and antioxidant status. TOS and TAC are a combination of oxidant and antioxidant parameters such as MDA, glutathione peroxidase and CAT. The TOS to TAC ratio gives OSI. As another parameter OSI reflects either increased oxidant status or decreased antioxidant capacity (Gokakin et al. 2013). Our results demonstrated that treatment with CP increased TOS and OSI while decreased TAC (Figure 8-10).

According to the biochemical results of our study, it is clear that CAR has substantial antioxidant effect against CP-induced oxidative stress. We determined that CAR administration has improved significantly SOD, CAT and GSH levels. Previous studies have reported that CAR has a protective effect against oxidative stress. Samarghandian et al. (2016) has suggested an ameliorative effect of CAR against immobilization stressed rats showing decreases in kidney SOD, CAT and GSH levels. Ahmadvand et al. (2016) reported that CAR has partial protective effect on gentamicin-induced renal toxicity. They showed a significant decrease in serum urea, but not in CRE levels. Bozkurt et al. (2014) found that CAR addition

decreased levels of MDA, TOS, and OSI and increased level of TAS in the rats treated alone with methotrexate to induce renal toxicity. El-Sayed et al. (2015) showed that CAR has partial protective effects against cisplatin-induced nephrotoxicity both biochemically and histologically. In their study levels of urea and CRE in serum and MDA in kidney are improved, while kidney GSH, SOD, CAT are not significantly changed in cisplatin plus CAR treated rats compared with cisplatin alone treated group.

The ability of CAR to enhance the levels of antioxidants along with its antilipidperoxidative activity suggests that this compound might be potentially useful in counteracting free radical mediated tissue damage in nephrotoxicity (Halliwell and Gutteridge 2007). Suganthi and Manpal (2013) reported that CAR is an efficient scavenger of free radicals including peroxy radicals, superoxide radicals, hydrogen peroxide and nitric oxide both in vitro and in vivo and its antioxidant activity is attributed to the presence of hydroxyl group. Eventhough we did not measure peroxynitrite levels in our study, CAR may also preclude formation of peroxynitrite by its ability of separately scavenging both reactive oxygen species and nitric oxide.

The effects of CP on kidney histology have already been demonstrated in previous studies (Abraham and Rabi 2011; Sayed-Ahmed 2010; Sugumar et al. 2007). Our histological results are consistent with these studies. In our study, addition of 10 mg/kg CAR provided a marked protection whereas 5 mg/kg CAR demonstrated a partial amelioration in CP-induced kidney damage. Other studies using CAR as a protective agent against different nephrotoxic drugs also showed significant improvements in kidney histology. Sayed-Ahmed (2010) reported that CP causes tubular necrosis and desquamation of lining epithelial cells with accumulation of eosinophilic material within tubular lumen in kidney. Furthermore, focal interstitial and tubular edema, connective tissue infiltration, cortical tubular vacuolization and glomerular

nephritis were also observed in his study. Histology results of Ahmadvand et al. (2016) suggested diminished tubular necrosis, but no change in leukocyte infiltration. Bozkurt et al. (2014) showed that CAR plus methotrexate treated rats exhibited decreased tubular degeneration compared to animals treated with methotrexate alone, but the difference was not statistically significant. El-Sayed et al. (2015) reported that CAR treatment improved cisplatin induced kidney damage significantly by reducing tubular necrosis, tubular cast formation and focal hemorrhage.

Altogether, these results suggest that CAR is able to act as a general antioxidant and protects biomolecules such as membrane lipids against free radical induced damage. In the present study, the increase in enzyme levels and antioxidant capacity in CAR injected groups together with CP can be evaluated as a response in order to decrease oxidative stress which occurs after CP injection. Microscopic examinations also confirmed the protective efficacy of CAR against kidney toxicity of CP.

In conclusion, current study supports the protective role of CAR against CP-induced renal damage. Thus, CAR may reduce the nephrotoxicity and kidney damage induced by CP for the patients undergoing chemotherapy. Therefore, CAR could be a potent candidate to be used concurrently as a supplement agent against nephrotoxicity of CP. Furthermore, it can also be used as a protective agent against other disorders of the urinary system. Further studies with different design are needed for clarification of the protective effects of CAR on CP-induced nephrotoxicity.

ACKNOWLEDGMENTS

This study was supported by grants (P.N: 201019007) from the Eskisehir Osmangazi University Scientific Research Project, Turkey. We thank Dr. Ahmet Mentese for his excellent technical assistance and Sedat Kacar for editing language.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1. Groups ($n=7$) and administrations by days.

	D1	D2	D3	D4	D5	D6	D7
Control	S	S	S	S	S	S	X
CP	S	S	S	CP	S	S	X
CP+5 CAR	CAR	CAR	CAR	CP+CAR	CAR	CAR	X
CP+10 CAR	CAR	CAR	CAR	CP+CAR	CAR	CAR	X
5 CAR	CAR	CAR	CAR	CAR	CAR	CAR	X
10 CAR	CAR	CAR	CAR	CAR	CAR	CAR	X

S: Saline

CP: Cyclophosphamide 100 mg/kg i.p.

CAR: Carvacrol 5 or 10 mg/kg i.p.

X: Sacrifice

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FIGURE LEGENDS

Figure 1: Kidney sections of control groups. Normal kidney histology in **a** and **b**: saline treated, **c**: 5mg/kg CAR treated and **d**: 10 mg/kg CAR treated rats. H-E. (Scale bars: a and b: 100 μm ; b and d 50 μm).

Figure 2: Kidney sections in CP-induced nephrotoxicity group. **a**: Mononuclear cell infiltration foci (*black arrows*), narrowing of Bowman capsular spaces (*empty arrows*) and degenerations in tubular cells in CP-induced nephrotoxicity group. **b**: Degeneration of some tubular cells, glomerular atrophy and compaction in glomerular cells (*empty arrows*) in CP-induced nephrotoxicity group. **c**: Some desquamations of tubular epithelial cells into tubular lumen (*empty arrows*) in 5 mg/kg CAR treated group. **d**: Nearly normal histological appearance in kidney corpuscles and tubules in 10 mg/kg CAR treated group. H-E. Scale bars: (a) 200 μm (b and d) 100 μm ; (c) 50 μm .

Figure 3: Comparison of serum malondialdehyde levels according to the groups ($n=7$, mean $\pm$ SD). The letters show significant difference ($p<0.05$) from **a**: control, **b**: CP, and **c**: CP+5 CAR groups. Only relevant differences are indicated.

Figure 4: Comparison of serum glutathione levels according to the groups ($n=7$, mean $\pm$ SD). The letters show significant difference ($p<0.05$) from **a**: control, **b**: CP, and **c**: CP+5 CAR groups. Only relevant differences are indicated.

Figure 5: Comparison of serum superoxide dismutase levels according to the groups ($n=7$, mean $\pm$ SD). The letters show significant difference ($p<0.05$) from **a**: control, **b**: CP, and **c**: CP+5 CAR groups. Only relevant differences are indicated.

Figure 6: Comparison of serum catalase levels according to the groups ($n=7$, mean $\pm$ SD). The letters show significant difference ($p<0.05$) from **a**: control, **b**: CP, and **c**: CP+5 CAR groups. Only relevant differences are indicated.

Figure 7: Comparison of serum creatinine levels according to the groups ($n=7$, mean $\pm$ SD). The letters show significant difference ($p<0.05$) from **a**: control, **b**: CP, and **c**: CP+5 CAR groups. Only relevant differences are indicated.

Figure 8: Comparison of serum total antioxidant capacities according to the groups ($n=7$, mean $\pm$ SD). The letters show significant difference ($p<0.05$) from **a:** control, **b:** CP, and **c:** CP+5 CAR groups. Only relevant differences are indicated.

Figure 9: Comparison of serum total oxidant status according to the groups ($n=7$, mean $\pm$ SD). The letters show significant difference ($p<0.05$) from **a:** control, **b:** CP, and **c:** CP+5 CAR groups. Only relevant differences are indicated.

Figure 10: Comparison of serum oxidative stress indexes according to the groups ($n=7$, mean $\pm$ SD). The letters show significant difference ($p<0.05$) from **a:** control, **b:** CP, and **c:** CP+5 CAR groups. Only relevant differences are indicated.

Draft

Figure 1.

128x96mm (300 x 300 DPI)

Figure 2.

128x96mm (300 x 300 DPI)

Figure 3.

64x38mm (300 x 300 DPI)

Figure 4.

64x38mm (300 x 300 DPI)

Figure 5.

64x38mm (300 x 300 DPI)

Figure 6.

64x38mm (300 x 300 DPI)

Figure 7.

64x38mm (300 x 300 DPI)

Figure 8.

64x38mm (300 x 300 DPI)

Figure 9.

64x38mm (300 x 300 DPI)

Figure 10.

64x38mm (300 x 300 DPI)