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Original Article

Satureja khuzistanica Jamzad essential oil prevents doxorubicin-induced apoptosis via extrinsic and intrinsic mitochondrial pathways

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Abstract

Background and purpose: In addition to hepato-cardiotoxicity, doxorubicin (DOX) also induces nephrotoxicity which is considered as the limiting factor for this drug in cancer therapy. The effect of carvacrol, the main active ingredient of Satureja khuzistanica Jamzad essential oil (SKEO), in the amelioration of DOX-induced cardiotoxicity is well established. The aim of the present study was to evaluate the possible protective effects of SKEO against DOX-induced nephrotoxicity.

Experimental approach: SKEO was intraperitoneally administered at 50, 100, and 200 mg/kg to male Wistar rats for 12 consecutive days. Five groups of animals including negative control (saline), vehicle (Tween® 20), SKEO50, DOX (at 8th day of treatment), and SKEO50 + DOX were assessed.

Findings/Results: Creatinine, urea concentrations, and caspase-3 activity significantly elevated in the serum of DOX treated group in contrast to other groups after injection of a single dose of DOX (20 mg/kg i.p.), however, SKEO reduced glutathione peroxidase and caspase-3 activity against other groups while SKEO + DOX was also significantly reduced caspase-3 activity against DOX group. Other biochemical markers changes were not significant. Immunohistochemical assessment unveiled that SKEO + DOX improved the activity of Bcl-2 family proteins (Bax and Bcl-2) and caspase-8 protein to the advantage of cell survival in both intrinsic mitochondrial and extrinsic pathway downstream to the terminal caspase-3 apoptotic molecule.

Conclusion and implications: It was concluded that SKEO could have influential effects against apoptosis induced by DOX, but not improperly ameliorate oxidative stress.

Keywords: Apoptosis; Carvacrol; Doxorubicin; nephrotoxicity; Satureja khuzistanica essential oil.

INTRODUCTION

Doxorubicin (DOX) is a chemo drug that belongs to the anthracyclines used for the treatment of some neoplasms. The mechanism of action of anthracyclines is the intercalation of DNA, the prevention of topoisomerase II, and the formation of free radicals (1). Although the positive effects of this chemotherapeutic drug are well determined on neoplastic cells, exclusively by activation of the apoptotic pathway, however, toxic effects of doxorubicin are still a major concern (2,3).

Reactive oxygen species (ROS) production in DOX prescription is the main factor in cell damages in many organs including cardiac muscles (4-7), hepatic cells (6,8,9), and renal tubular cells (6,10-13) in which the well-defined side effect is cardiotoxicity. In this regard, many researchers exerted to reduce the side effects of DOX-induced organ toxicity with a variety of antioxidant agents. Of these candidate antioxidants with the purpose of protecting the cells from DOX-induced cytotoxicity, coenzyme Q10 (10), proanthocyanidins (12), and nicotinamide (11) are more common.

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Carvacrol (2-methyl-5-[1-methylethyl]-phenol) is a monoterpenoid compound found in essential oils of aromatic plants such as oregano, thyme, pepperwort, and wild bergamot (14,15) and also found in Satureja khuzestanica Jamzad (Marzeh khuzestani in Persian, the family of Lamiaceae, the subfamily of Nepetoidae). It is an endemic plant that grows in the southern parts of Iran (16). Carvacrol has numerous biological and pharmacological activities including antioxidant, anti-inflammatory, anticancer, antibacterial, antifungal, hepatoprotective, spasmyolic, and vasorelaxant, both in vitro and in vivo (15,17,18). Recently, a protective effect of carvacrol by reducing cisplatin-induced nephrotoxicity has been confirmed (19). Therefore, the present study was designed to investigate the carvacrol antioxidant effect in more detail on oxidative stress-related changes by DOX-induced nephrotoxicity.

MATERIALS AND METHODS

Chemicals
Satureja khuzistanica essential oil (SKEO) (Carvacrol, CAR 83.8%, was determined as the main active ingredient by gas chromatography-mass analyzer) was obtained from Khorraram Pharmaceutical Complex, Khorramabad, Iran. Doxorubicin was obtained from the Ribodoxo Company (Germany). Blood urea nitrogen (BUN) and creatinine kits were provided from Parsazmoon Company (Tehran, Iran). The nitric oxide (NO) determination kit was purchased from Kiazist life sciences (Iran) and caspase-3 activity was supplied from Biovision Company (USA).

Animals
Sixty-three adult male Wistar rats weighing 200-250 g were obtained from the Razi herbal medicine research center of Lorestan University of Medical Sciences. All experimental procedures were performed in accordance with the ethical guidelines for laboratory animals’ research with approval number 201924, confirmed by the ethical committee of Lorestan University.

Experimental design
The rats were kept in a room with controlled conditions for laboratory animals, including a temperature of 23 ± 2 °C, the humidity of 50%, 12/12-h light/dark cycles, and free access to food and water, then were divided into 9 groups, 7 each, as follows:
(a) Negative control group: rats were given 0.9% NaCl intraperitoneally (IP) for 12 successive days as a vehicle; (b) Vehicle group which received daily Tween® 20 (1%, IP) for 12 consecutive days as an emulsifier for SKEO solvent; (c-e) SKEO emulsified with Tween® 20 (1%) was administered (IP) at 50 mg/kg, (SKEO 1) 100 mg/kg (SKEO 2), and 200 mg/kg (SKEO 3) for sequential days; (f) DOX (20 mg/kg, IP) injected in a single dose on the 8th day of treatment; (g-i) SKEO-treated DOX groups 12 days (SKEO) and 8th day (DOX), injected in 50 (SKEO 1 + DOX), 100 (SKEO 2 + DOX) and 200 (SKEO 3 + DOX).

According to a study by Abbasloo et al. (20), doses of 50, 100, 200 mg/kg were chosen. In our experiment, we found out that more than 50 rats were poisoned and died at doses of 100 and 200 mg/kg after second attempts. Therefore, only 50 mg/kg dose was selected as the maximal safe dose.

Serum and tissue preparation
On the 12th day, the rats were anesthetized with chloroform then sacrificed by taking blood through cardiac puncture. The blood was clotted, serum was separated and transferred to tubes. The serum was collected and stored at -20 °C. Both kidneys were removed. The right kidney was immersed in a 10% formalin buffered solution for histopathological and immunohistochemical analysis and the left one was stored at -70 °C for biochemical analysis.

Biochemical analysis
Serum creatinine and urea were determined spectrophotometrically (WPA, Cambridge, UK) according to the manufacturer’s instructions for evaluation of renal function.

Tissue preparation
Rat kidneys were thawed and manually homogenized in cold phosphate buffer (0.1 M, pH 7.4) containing 5 mM
ethylenediaminetetraacetic acid (EDTA) using liquid nitrogen and protein content of tissue homogenates was determined by the Bradford method with bovine serum albumin as a standard (21).

**Measurement of glutathione peroxidase activity**

The activity of glutathione peroxidase (GPx) was evaluated with Randox® GPx detection kit (UK) according to the manufacturer’s instructions, as described previously (22). GPx catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of GSH reductase and NADPH, the oxidized GSH is immediately converted to the reduced form with concomitant oxidation of NADPH to NADP+. The decrease in absorbance was measured spectrophotometrically (S2000 UV model; WPA, Cambridge, UK) against blank at 340 nm. One unit (U) of GPx was defined as 1 µmol of oxidized NADPH per min per mg of tissue protein. The GPx activity was expressed as unit/mg of tissue protein (U/mg protein).

**Measurement of lipid peroxidation**

The amount of lipid peroxidation was indicated by the content of thiobarbituric acid reactive substances (TBARS) in the kidney. Tissue TBARS was determined by following the production of TBARS. Briefly, 40 µL of supernatant was added to 40 µL of 0.9% NaCl and 40 µL of deionized H₂O₂, resulting in a total reaction volume of 120 µL. The reaction was incubated at 37 °C for 20 min and stopped by the addition of 600 µL of cold hydrochloride acid (0.8 mol/l), containing 12.5% trichloroacetic acid. Following the addition of 780 µL of 1% TBA, the reaction was boiled for 20 min and cooled at 4 °C for 1 h. In order to measure the amount of TBARS produced by the homogenate, the cooled reaction was spun at 15000 g in a microcentrifuge for 20 min and the absorbance of the supernatant was spectrophotometrically read at 532 nm, using an extinction coefficient of 1.56 × 10⁵ /mol cm. The blanks for all of the TBARS assays contained an additional 40 µL of 0.9% NaCl instead of homogenate as just described. TBARS results were expressed as nmol/mg tissue protein (23).

**Measurement of catalase activity**

Tissue catalase (CAT) activity was assayed using the method described by Kheradmand et al. (24). The reaction mixture (1 mL) consisted of 50 mmol/L potassium phosphate (pH 7.0), 19 mmol/L H₂O₂, and a 25 µL of tissue sample homogenate. The reaction was initiated by the addition of H₂O₂ and absorbance changes were measured at 240 nm (25 °C) for 30 s. The molar extinction coefficient for H₂O₂ is 43.6/mol cm. The CAT activity was expressed as the unit that is defined as 1 mol of H₂O₂ consumed per min per mg of tissue protein (unit/mg protein).

**NO assay**

The amount of total stable nitrite, the end product of NO generation, was determined by a colorimetric method via Kiazist life sciences kit, as described by Kim et al. (25). In brief, 50 µL of tissue homogenate was mixed with 100 µL of Griess reagent (1 % sulfanilamide, 0.1 % naphthylethelenediamine dihydrochloride, and 2.5 % H₃PO₄), and 1850 µL distilled water. After 10 min of incubation at room temperature, absorbance was read at 540 nm. The blank was prepared with the same method however, instead of 50 µL of the tissue homogenate, 50 µL of distilled water was applied. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve and results were expressed as mmol/mg tissue protein.

**Caspase-3 activity**

The caspase-3 activity was measured using a caspase-3 colorimetric assay kit according to the manufacturer’s instructions (Biovision, Mountain View, USA) by a spectrophotometer, as described by Alirezaei et al. (21). The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide by caspase-3, resulting in the release of the p-nitroaniline moiety. p-Nitroaniline has a high absorbance at 405 nm (εM =10.5). The concentration of the p-nitroaniline released from the substrate is calculated from the absorbance values at 405 nm. Enzyme activity was defined as produced µmole of p-nitroaniline (ng/mg) of tissue protein.
Histopathological and immunohistochemical procedures

For assessment of renal changes, formalin-fixed kidneys were sectioned at 5 µm and stained with hematoxylin and eosin. Acute tubular necrosis (ATN) of the epithelium was assessed in the full thickness of the cortex, which divided into three distinct areas including the subcapsular region (supracortex), cortex, and corticomedullary junction (subcortex) were scored according to the degree of epithelial damage using a zero through five grading system: 0, no lesion; 1, ≤ 10% ATN; 2, 11% to 25% ATN; 3, 26% to 50% ATN; 4, 51% to 75% ATN; and 5, 76% to 100% ATN. Moreover, the presence of hyaline cast was evaluated according to the percent of tubules involved in 10 low power fields.

Three µm dewaxed and rehydrated renal tissues were immersed in a target retrieval solution (pH 9.0) and boiling water bath for 20 min at 98 °C to delivered unmasked antigens. Then the sections were treated with 3% H2O2 in phosphate-buffered saline (PBS) for 15 min to block endogenous peroxidase. To prevent nonspecific-background staining the sections were incubated with 5% normal rabbit sera in PBS for 20 min. Sections were incubated 1 h with polyclonal rabbit anti-Casp-3 (orb 10237, Biorbyt, UK) at 1:50 dilution, Casp-8 (orb 10664, Biorbyt, UK) at 1:100, proliferating cell nuclear antigen (PCNA) (orb 128497, Biorbyt, UK) at 1:50, B-cell lymphoma (Bcl)-2 (orb 10173, Biorbyt, UK), at 1:50 and Bcl-2-associated X protein (Bax) (sc 7480, Santa Cruz, USA) at 1:50, then incubated for 20 min in biotinylated goat anti-rabbit IgG (prediluted, Biocare, USA), followed by incubation with streptavidin horseradish peroxidase (prediluted; Biocare, USA) for 20 min. The antibody binding sites were visualized through reaction with DAB solution. Finally, sections were counterstained with Mayer's Hematoxylin (Bio Optica, Italy).

H score assessment was used randomly in high power field for staining intensity scored 0, negative; 1, weak; 2, intermediate; and 3, strong and also for percentage of cells labeled (no labeling = -/+; labeling ≤ 10% = +1, 10 up to 50% = +2, and more than 50% = +3).

Statistical analysis

Statistical Package for the Social Science (SPSS, version 18; SPSS Inc., Chicago, Illinois, USA) was used for the analysis of data. Data were presented as mean ± standard error of the mean (SEM) and compared between groups using one-way ANOVA followed by a post-hoc Tukey’s test. A P-value ≤ 0.05 was considered statistically significant.

RESULTS

Effects SKEO on body weight and organ weights

There were no significant changes in the kidney/body weight ratio by the administration of 50 mg/kg SKEO, and DOX treated groups in comparison to the controls. All rats in the controls, SKEO 50, DOX, and DOX + SKEO 50 were alive during the experiment. However, mortality was observed in animals of SKEO 100, 200, DOX + SKEO 100, and DOX + SKEO 200 (LD50).

Effects of SKEO on urea and creatinine in DOX-treated rats.

DOX significantly, increased urea of plasma in the DOX group in comparison with SKEO (P < 0.001) while there was no significant difference between control and SKEO-DOX groups (P > 0.05; Fig. 1). Creatinine as renal failure index significantly increased in the DOX group in comparison with the control and (P < 0.05). However, SKEO decreased creatinine concentration insignificantly when compared to SKEO-DOX group (P > 0.05, Fig. 2)

Fig. 1. Effects of SKEO and DOX therapy on urea in rats.

**P < 0.01 Indicates significant differences in comparison with SKEO. SKEO, Satureja khuzestanica essential oil; DOX, doxorubicin.
**Effects of SKEO on renal GPx, CAT, lipid peroxidation, NO, and caspase-3 activity in DOX-induced nephrotoxicity**

GPx activity decreased significantly in SKEO group when compared with control, vehicle, and DOX groups (\( P < 0.05 \), Fig. 3).

SKEO scavenged ROS and induced activity of GPx. Therefore, there was no significant difference among the SKEO group in comparison with the control and DOX groups (\( P > 0.05 \)). Although SKEO via quenching free radicals decreased CAT activity in SKEO and SKEO-DOX groups, these reductions were not significantly different than to DOX group (\( P > 0.05 \); Fig. 4).

DOX as a peroxidative agent-induced lipid peroxidation shown by malondialdehyde (MDA) elevation. However, this increment was not significant while SKEO treatment could decrease MDA concentration in SKEO and SKEO-DOX groups (\( P > 0.05 \), Fig. 5).

SKEO treatment in the SKEO group as insignificantly decreased NO concentration. It seems NO was not acting as an important inflammatory mediator in renal failures induced by DOX (\( P > 0.05 \), Fig. 6).
Caspase-3 activity as an apoptosis factor decreased in SKEO and SKEO-DOX groups (\(P < 0.001\)) in contrast to DOX group. However, caspase-3 activity increased in the DOX group when compared with vehicle control groups (\(P < 0.001\), Fig. 7). Also there were no differences between control, vehicle and SKEO-DOX groups (\(P > 0.05\)). Indeed, \textit{SKEO} in SKEO-DOX group as significantly decreased caspase-3 activity and apoptosis.

\textbf{Effects of SKEO on renal histopathology in DOX-treated rats.}

The severity of ATN and the level of hyaline cast formation in untreated nephrotoxic animals significantly increased following DOX administration compared to the controls, SKEO, and SKEO50 + DOX groups (\(P \leq 0.0001\)). No significant changes were seen among other groups (\(P = 0.1\)). Moreover, glomerular changes were also insignificant following DOX administration.

\textbf{Effects of SKEO on the immunoexpression of apoptotic markers and PCNA}

The immunoexpression of Bax, Casp-8, Casp-3, Bcl-2, and PCNA are depicted in Fig. 8. Following DOX administration, immunolabeling of pro-apoptotic antibodies (Bax, Casp-8, and Casp-3) significantly enhanced in the DOX treated group in comparison to the control and SKEO groups. The immunopositive cells were restricted to glomeruli and only very few tubular cells. However, Bcl-2 immunoreaction decreased in DOX treated group. SKEO-DOX administration inhibited the expression of Bax, Casp-8, and Casp-3 and increased Bcl-2 immunoreaction. There was no difference between PCNA expressions in DOX and DOX-SKEO groups, while in control and SKEO groups nuclear reaction was prominently lower.

Fig. 6. Effects of SKEO and DOX therapy on NO in kidney of rats. There are no significant differences amongst groups (\(P > 0.05\)). SKEO, \textit{Satureja khuzestanica} essential oil; DOX, doxorubicin; NO, nitric oxide.

Fig. 7. Effects of SKEO and DOX therapy on caspase 3 in the kidney of rats. Caspase-3 activity decreased in SKEO and SKEO-DOX groups (\(*\ast\ast\ast P < 0.001\)) compared to DOX. \(*\ast\ast\ast P < 0.001\) indicates significant difference compared with negative and vehicle groups. SKEO, \textit{Satureja khuzestanica} essential oil; DOX, doxorubicin.
Fig. 8. Effects of SKEO on the immunoexpression of apoptotic markers and PCNA in Rat Kidney. (A-D) Immunohistochemistry of Bax protein. A low population of only a few epithelial cells and glomeruli are expressing proapoptotic marker in control and SKEO group. Massive expression of antibody in both glomeruli and tubular cells in DOX group and restricted positive area in DOX + SKEO group. (E-H) Immunolabeling of Caspase-8. Immunopositivity limited to some glomeruli in control and SKEO group. The large population of tubular epithelial cells and glomeruli has immunoreaction to marker in DOX group but decreased its expression in DOX + SKEO group. (I-L) Immunoreaction of Caspase-3 antibody. Similar to casp-8, the expression of cap-3 is limited to glomeruli in control and SKEO group. Diffuse strong cytoplasmic expression in tubular cells in DOX group and lower immunolabeling in in DOX + SKEO treated rats. (M-P) Bcl-2. Highly immunoreaction of antibody is present in tubular cells in control and SKEO group. Immunopositive cells prominently reduced in numbers and intensity in DOX but were more intensified in DOX + SKEO group. (Q-T) PCNA protein. Very few epithelial cells nuclei show strong staining to antibody specified with arrows in control and SKEO group. More nuclei are immunostained with the marker in DOX and DOX + SKEO treated rats. SKEO, Satureja khuzistanica essential oil; DOX, doxorubicin; Bcl-2, B-cell lymphoma; Bax, Bcl-2-associated X protein; PCNA, proliferating cell nuclear antigen.

DISCUSSION

Renal tissue is a susceptible organ to DOX and its metabolites which could induce renal impairment by several mechanistic effects. Although part of the cell damages might be well understood, the other mechanistic injuries remained obscure. It is well realized now that DOX-treated animals show side effects by ROS formation and subsequently oxidative stress injury comes along with stimulation of inflammatory response and apoptosis (4-9,17). One of the DOX-induced nephrotoxicity mechanisms is the suppression of superoxide...
dismutase, catalase, and GPx activities. Also, GSH depletion might lead to an excessive increase of lipid peroxidation marker such as MDA (11,17). According to shreds of evidence, GSH as a cofactor of glutathione peroxidase (GPx) has the main role in the cellular defense system against lipid peroxidation. GPx as a main antioxidant enzyme decomposes H₂O₂ and prevents ROS production. GSH is preserved in cells as a reduced form and converted to oxidized GSH as an oxidized form in cellular oxidative conditions (17,26,27).

The decrease in GPx activity in SKEO-treated rats indicates the scavenging role of SKEO to trap ROS, and subsequently decrease of GPx activity. The observed protective effect of SKEO in this study was associated with the reduction of MDA. It seems that the increase of CAT and GPx activities (though insignificant) in the DOX group may be a compensatory mechanism that was not able to decrease lipid peroxidation in rats. Although both GPx and CAT activities were lower in SKEO-DOX group against DOX treated rats, SKEO significantly was unable to suppress oxidative stress and prevent lipid peroxidation in the kidney of rats.

The former studies have focused on the oxidant/anti-oxidant imbalance and therefore the generation of free radicals that terminate the tissue damage (6,17,28). However, there is little information on how the apoptosis signaling pathway is triggered by DOX?

It is clear that both glomerular apparatus and tubular structures are unprotected against DOX toxicity. In histopathological investigations, massive destruction of glomerular tufts including, hypertrophy of glomeruli, tubular disorganization, and necrosis are well described (28). High levels of BUN and creatinine indicating of renal insufficiency in the DOX treated group and as mentioned earlier, it is related to free radicals generation and subsequently membrane lipid peroxidation, MDA production, and cellular damage as the main path (28).

But what will happen in the next, or in parallel with impairment of enzymatic defense mechanism, is DNA fragmentation in which the pro-apoptotic molecules in intrinsic pathways of apoptosis, including Bax, Bad, apoptotic protease activating factor-1 (Apaf-1), and caspase cascade associated with cytochrome c are highly expressed, whereas anti-apoptotic molecules like Bcl-xl and Bcl-2 are inactivated. Therefore, it concluded that mitochondrial damage and loss of its membrane function is the main target for DOX to initiate the intrinsic pathway of apoptosis. Briefly, after translocation of p53 and Bax from the cytosol to the outer membrane of mitochondria, cytochrome c is released which in turn activates APAF-1 and then it binds to caspase-9 and the apoptosome complex is formed. Inactive pro-caspase-3 is converted to active caspase-3 by apoptosome and DNA degradation will initiate (28-30). Caspase-3 decreased significantly in the SKEO group but conversely, in the DOX-treated rats, caspase-3 activity was significantly increased in our results. Further evidence to support this premise comes from the finding that activated caspase-3 is controlled by SKEO in the SKEO-D group. The results of the present study indicated the anti-apoptotic role of SKEO versus the apoptotic role of DOX.

In similar works cisplatin also showed the same effects as DOX that is associated with free radicals formation and renal cell protein and lipid peroxidation (16). Like DOX's cell injury mechanisms, the intrinsic pathway of apoptosis is induced by cisplatin through the upregulation of p53 as an upstream molecule of Bax and terminates to caspase-3 (14,31,32).

PCNA is a p53 dependent molecule that plays two different roles, including DNA replication (when p53 is decreased) and DNA repair (when p53 is increased). p21 is a dependent p53 protein that binds with PCNA in order to arrest the cell cycle and thereafter the potential role of PCNA in DNA repair is started in CP treatment animals. However, PCNA induction by cisplatin might reveal also the anti-apoptotic effect in proximal tubular cells (14,33,34). In the present study, it was assumed that DOX has a similar effect on the protective role of PCNA upregulation.

Our study indicated that carvacrol doesn’t have sufficient protective effects against oxidative stress, which was induced by DOX. It could not improve the antioxidant levels of GPx, CAT, and consequently, prevent the MDA peroxidation level through inhibiting free
radicals production and therefore stabilizes the cell membrane. However, carvacrol could reduce the cellular damages of DOX by preventing of intrinsic (Bax), extrinsic (caspase-8), and common downstream caspase-3 apoptotic pathways. In one study, carvacrol reduced the cisplatin side effects by anti-apoptotic effect through activated inhibition of p53, p21, and PCNA proteins and reinforces cell cycle reactivation through cyclins-cyclin dependent kinases (14).

CONCLUSION

We are concluded that the border between drug efficacy and toxicity of SKEO is very limited. In this study low dose of SKEO had anti cytotoxic effects and a mild degree of antioxidative properties against DOX-treated animals. However, with an increase of SKEO dose (100 and 200 mg/kg) 50% of rats were succumbed. Although general consensus believed that SKEO has very potent cytoidal effects on proliferative cells like malignant neoplastic cells but its protective effect against oxidative stress exclusively on renal epithelial cells is controversial. More investigation is suggested for SKEO cytotoxic mechanisms in normal animals.

CONFLICT OF INTEREST STATEMENT

All authors declared that no conflict of interest in this study.

AUTHORS’ CONTRIBUTION

O. Dezfulian contributed to the research idea and study design. O. Dezfulian, M. Aliarezaei, and A. Al Seyedan the acquired data. O. Dezfulian and M. Aliarezaei analyzed the data. O. Dezfulian wrote the paper and all the authors read and approved the final manuscript.

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