Resveratrol Protects Sepsis-Induced Oxidative DNA Damage in Liver and Kidney of Rats

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Background: The increases of free radicals have been proposed to be involved in the pathogenesis of sepsis, which leads to multiple-organ dysfunction syndromes. The uses of antioxidants as a complementary tool in the medical care of oxidative stress-related diseases have attracted attention of researchers. Resveratrol (RV) has suggested being antioxidant, anti-proliferative, and anti-inflammatory effects in various experimental models and clinical settings.

Aims: This study was undertaken to evaluate the protective effects of RV on oxidative DNA damage induced by sepsis in the liver and kidney tissues of Wistar albino rats.

Study Design: Animal experimentation.

Methods: Four experimental groups consisting of eight animals for each was created using a total of thirty-two male Wistar albino rats. Sham group was given 0.5 mL of saline intra-peritoneal (ip) only following laparatomy. Sepsis group was given 0.5 mL saline ip only following the induction of sepsis. RV-treated group was given a dose of 100 mg/kg ip RV in 0.5 mL saline following laparatomy. RV-treated sepsis group was given 100 mg/kg ip RV in 0.5 mL saline following the induction of sepsis. A model of sepsis was created by cecal ligation and puncture technique. In the liver and kidney tissues, oxidative stress parameters (malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPX)) and a proinflammatory cytokine (tumor necrosis factor alpha (TNF-alpha)), were evaluated spectrophotometrically and DNA damage was determined by the alkaline single cell gel electrophoresis (comet assay) technique using formamidopyrimidine DNA glycosylase protein.

Results: In the RV-treated sepsis group, the levels of MDA and TNF-alpha were lower and GSH levels, SOD and GPX activities were higher than in the septic rats (p<0.05). RV treatment significantly reduced the sepsis-induced oxidative DNA damage in the liver and kidney cells (p<0.05).

Conclusion: It is suggested that RV treatment might reduce the sepsis-induced oxidative DNA damages in sepsis-related diseases; however, there is a need for more studies to clear up the protective mechanisms of RV against sepsis.

Keywords: Resveratrol, sepsis, tumor necrosis factor alpha, oxidative stress, DNA damage, alkaline single cell gel electrophoresis

Sepsis, a serious medical condition, causes mortality and morbidity in intensive care units. It is characterized by the production of proinflammatory cytokines and reactive oxygen species (ROS) generated by macrophages and neutrophils after infection (1). Oxidative stress occurs due to the excessive formation of ROS and inadequate antioxidant defense system in sepsis. ROS is a crucial issue in the progression of sepsis-induced multi-organ damages, which leads to the inflammatory effects, lipid peroxidation, and DNA damage (2). Antioxidants maintain the redox balance in sepsis. In many studies, it was shown that antioxidant status was reduced in septic patients and antioxidants such as vitamin C, vitamin E, and other natural antioxidants ameliorated the oxidative stress markers changed in sepsis. Antioxidants can also interfere

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with inflammatory responses by regulating proinflammatory and inflammatory cytokines and endotoxins, thus modulating cellular activation (1,2).

Resveratrol (3,4′,5-trihydroxystilbene) (RV) is a radical-scavenger. It ameliorates antioxidant status, reduces the expression of inflammatory biomarkers and prevents or cures cardiovascular diseases and improves microcirculatory disorders (3,4). RV, a natural antioxidant, has been suggested to reduce DNA damage and oxidative organ injury (5). Several studies demonstrated the hepatoprotective effects of RV *in vivo* (6,7). RV (10 mg/kg body weight (bw)) pretreatment was found to suppress the lipid peroxidation in the kidneys of ferric nitrilotriacetate-treated rats (5). The enzyme Fpg is the major enzyme for removal of oxidized purines in bacteria and has been recommended to determine the oxidative DNA damage, in particular 8-hydroxyguanine in conjunction with the comet assay (8).

This present study was undertaken to investigate the ameliorative properties of RV on the oxidative DNA damage induced by sepsis in the liver and kidney tissues of Wistar albino rats. Our hypothesis is that RV reduces or prevents the sepsis-induced oxidative stress and DNA damage in the main target tissues. This study may clarify the possible mechanisms of RV on the sepsis-induced oxidative DNA damage *in vivo*.

MATERIALS AND METHODS

Animals

In this study, a total of 32 male Wistar albino rats weighing 200-300 g (3 months old) were housed in plastic cages with stainless-steel grid tops. Before the experiments, they were kept under a controlled environment regarding humidity (50%), temperature (23±2°C), and light (12-hour light-dark cycle). All rats were fed with standard laboratory animal feed and allowed to access drinking water and *ad libitum* feed before and after operation. All surgical procedures were carried out under anesthesia with intraperitoneal (ip) injection of 90 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı, Warner-Lambert, İstanbul, Turkey) (9). The local Animal Ethical Committee (Hacettepe University Animal Ethical Committee) of this study was approved.

Experimental design

A model of sepsis created by cecal ligation and puncture (CLP) technique was applied to rats as described previously (10,11). Four experimental groups consisted of eight animals for each was carried out. Sham group was given 0.5 mL of saline intraperitoneal (ip) only following laparotomy. In the sham group applied laparotomy; the cecum without ligated or perforated was manipulated. Sepsis group, where only CLP

was performed, was given 0.5 mL saline ip following the induction of sepsis. RV-treated group was immediately given a dose of 100 mg/kg bw ip RV in 0.5 mL saline following laparatomy. RV-treated sepsis group was immediately given 0.5 mL of a solution containing 100 mg/kg bw ip RV following the induction of sepsis. The treatment dose of RV was selected from the study of Della-Morte et al. (12). At the studied dose, 100% of the animals remained alive. In our previous studies with the phenolic compounds pycnogenol®, ferulic acid, and rosmarinic acid, the dosages of 100 mg/kg bw ip were also used in the experimental sepsis model (13-15). In these studies evaluated independently form the same local animal ethic approval to reduce animal numbers according to the principles of 3Rs, sham and sepsis groups were common; thus the data from sham and sepsis groups were the same in these studies carried out at the same time (13-16).

All animals were decapitated under the anesthesia, 24 hours following the treatment. Blood samples obtained from cardiac puncture were taken in preservative-free heparin tubes. The tissues of liver and kidney were gently removed as a whole. The liver and kidney tissues were cut into several pieces to perform the structural analysis of the tissues and to determine of antioxidant parameters and DNA damage. The organs were examined for the changes in size, color, and texture, in which there were no changes in the histological structures of the liver and kidney tissues.

Determination of oxidative stress parameters

The liver and kidney tissues were extracted following a homogenization and sonication procedure (17). The tissue samples were rinsed with cold phosphate buffered saline (PBS) solution, pH 7.4 to remove any red blood cells and clots. The tissues were homogenized in 5 volumes of fractionation buffer (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, cOmplete Protease Inhibitor Cocktail (Roche Diagnostics; Indianapolis, IN)] using a Potter-Elvehjem homogenizer. A 10% tissue homogenate was centrifuged at 10000xg for 15 min at 4°C. The homogenized tissue samples were stored at -80°C until the analysis. The supernatant was deproteinized for GSH analysis according to the manufacturer's instructions before storing.

The reduced glutathione (GSH) levels, superoxide dismutase (SOD) and glutathione peroxide (GPX) enzyme activities in these tissues were evaluated spectrophotometrically using GSH , SOD, and GPX assay kits (Cayman Cemicals Co.; Ann Arbor, MI, USA) at 440, 340, and 405 nm, respectively, according to the manufacturer's instructions. Results were given as mmol/min/mg tissue.

The level of malondialdehyde (MDA), an indicator of lipid peroxidation, was analyzed by thiobarbituric acid-reactive substances (TBARS) assay (18). The results were given as nmol/g tissue.

Determination of plasma tumor necrosis factor alpha (TNF-alpha) levels

The plasma from whole blood sample was kept at -80°C until the time of analysis. The level of TNF-alpha was measured at 30 min in duplicate at 450 nm. The protocol from the manufacturer's instructions using highly sensitive enzyme-linked immunosorbent assay (ELISA) kits containing Invitrogen KRC0011 (USA) was performed. The results were given as pg/mL.

Determination of DNA damage

Standard and formamidopyrimidine-DNA glycosylase (Fpg) modified comet assay described by Singh et al. (19), as further described by Anderson et al. (20) and Collins et al. (8) was performed. The single-cell suspension from the liver and kidney tissue samples was prepared according to a standard procedure (21). Each organ (about 0.2 g) was gently placed in 1 mL cold HBSS containing 20 mM EDTA-Na$_2$ and 10% dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich Chemicals (Sigma-Aldrich Chemicals; St. Louis, USA) in a petri plate and sliced into small parts using a steel scissor. The pieces were let to settle and the single-cell suspension in the supernatant containing was used. The number of cells was adjusted to $2x10^6$ cells/mL. The cells were embedded on agarose gel, lysed, and fragmented DNA strands revealed by the alkaline electrophoresis to create a comet. The details of the alkaline comet assay with and without Fpg protocol was published previously (9). After electrophoresis, the slides were neutralized and then dried. In the dried microscopic slides, DNA damage was investigated at 40x magnification. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd.; Liverpool, UK) to determine DNA damage. Results were presented as DNA tail length, DNA tail intensity (% tail DNA), and DNA tail moment. One hundred cells from each of 2 replicate slides were counted. The difference of DNA damage between the Fpg-modified comet assay and the standard comet assay indicated the oxidative DNA damage in a single cell.

Statistical analysis

Statistical analysis was performed using the software programs Statistical Package for the Social Sciences version 17.0 (SPSS Inc.; Chicago, IL, USA). The Shapiro-Wilk test to check the normality of data distribution and the Levene test to verify the homogeneity of the variance were used. The differences between the means of data were compared by the one way variance analysis test. Dunnet's T3 test or LSD test was used for the determination of post hoc analysis of group differences related to the variance between groups. The mean±standard deviation of the results was presented. P value less than 0.05 was accepted as statistically significant.

RESULTS

Oxidative stress parameters

Glutathione and MDA levels and SOD and GPX activities for the liver and kidney tissues are shown in Table 1 and 2, respectively. Hepatic and renal GSH levels, SOD and GPX activities decreased significantly in the septic rats versus to the sham operated group (p<0.05). Unlike hepatic and renal GSH levels, hepatic SOD and GPX activities, and renal GPX activities in the RV-treated sepsis group were significantly lower than in the sham group (p<0.05). Hepatic and renal GSH levels and SOD and GPX enzyme activities significantly increased in the RV-treated sepsis group when compared to the sepsis group (111% and 281% for liver and renal GSH, respectively; 12.2% and 40.3% for liver and renal SOD; 167% and 52.8% for liver and renal GPX) (p<0.05). Hepatic and renal MDA levels significantly increased in the sepsis group when compared to the sham group (p<0.05). Hepatic and renal MDA levels significantly decreased in the RV-treated sepsis group when compared to the sepsis group (37.9% and 50.6% for liver and renal MDA, respectively) (p<0.05). Hepatic MDA level in the RV-treated sepsis group was significantly higher than in the sham group (p<0.05).There were no significant differences in all hepatic and renal oxidative stress parameters between the sham and the RV-treated groups.

Plasma TNF-alpha levels

The result of plasma TNF-alpha levels is given in Figure 1. Plasma TNF-alpha levels significantly increased in the both sepsis and RV-treated sepsis groups when compared to the sham group (p<0.05). However, it significantly decreased in the RV-treated sepsis group when compared to the sepsis group (54.6% for TNF-alpha) (p<0.05). There was no significant difference between the sham and the RV-treated groups.

DNA damage in the liver and kidney tissues

DNA damage in the liver and kidney cells are shown in Figures 2a-c and 3a-c, respectively. In the standard comet assay, there were no statistically significant differences in terms of DNA tail length (TL), DNA tail intensity (TI), and DNA tail moment (TM) in the liver and kidney cells between the sham and the RV-treated groups. All DNA damage parameters in the sepsis group were significantly higher than in the sham group (p<0.05) and RV treatment significantly reduced DNA damage induced by sepsis in these tissue cells (24.1%, 45.2%, and 45.0% for TL, TI, and TM in the liver, respectively, and 18.5%, 39.1%, and 43.4% for TL, TI, ad TM in

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TABLE 1. Oxidative stress parameters in the liver tissues

	GSH levels (nmol/mg tissue)	MDA levels (nmol/g tissue)	SOD activity (mmol/min/mg tissue)	GPX activity (nmol/min/mL)
Sham group	3.52±0.40	11.32±2.73	164.6±6.91	86.58±4.69
Sepsis group	1.05±0.30*	24.80±4.36*	131.6±5.86*	20.34±3.31*
	p<0.001*	p=0.004*	p<0.001*	p<0.001*
RV-treated group	3.72±0.44#	10.50±1.95#	161.0±10.54#	78.40±2.77#
	p<0.001#	p=0.004#	p=0.007#	p<0.001#
RV-treated sepsis group	2.22±0.53*#	15.40±2.80#	147.6±6.27*#	54.30±4.16*#
	p=0.014*	p=0.026#	p=0.019*	p<0.001*
	p<0.022#		p=0.017#	p<0.001#

*p<0.05, compared to the sham group, #p<0.05, compared to the sepsis group. The results were given as the mean±standard deviation.
RV: resveratrol; GSH: reduced glutathione; MDA: malondialdehyde; SOD: superoxide dismutase; GPX: glutathione peroxide

TABLE 2. Oxidative stress parameters in kidney tissues

	GSH levels (nmol/mg tissue)	MDA levels (nmol/g tissue)	SOD activity (mmol/min/mg tissue)	GPX activity (nmol/min/mL)
Sham group	2.13±0.47	13.81±0.80	148.0±15.6	123.78±10.99
Sepsis group	0.47±0.24*	32.20±4.35*	102.2±12.1*	46.94±6.42*
	p<0.000*	p<0.001*	p=0.005*	p<0.001*
RV-treated group	2.22±0.25#	12.72±1.27#	151.8±14.9#	121.86±9.96#
	p<0.001#	p<0.001#	p=0.003#	p<0.001#
RV-treated sepsis group	1.79±0.14#	15.92±1.43#	143.4±8.70#	71.74±4.16*#
	p<0.001#	p<0.001#	p<0.000*	p=0.001*
		p=0.002#	p=0.001#	

*p<0.05, compared to the sham group, #p<0.05, compared to the sepsis group. The results were given as the mean±standard deviation.
RV: resveratrol; GSH: reduced glutathione; MDA, malondialdehyde; SOD: superoxide dismutase; GPX: glutathione peroxide

the kidney, respectively) (p<0.05). In the Fpg-modified comet assay, there were no statistically significant differences in all DNA damage parameters of the liver and kidney cells between the sham and the RV-treated groups. These parameters in the septic rats were found to be significantly higher than in the sham group (p<0.05) and RV treatment significantly reduced sepsis-induced DNA strand breakages in the liver and kidney cells (28.9%, 40.6%, and 53.3% for TL, TI, and TM in the liver, respectively, and 43.7%, 50.4%, and 58.8% for TL, TI, and TM in the kidney, respectively) (p<0.05). The enzyme-sensitive sites, more prone for the strand breaks and indicating DNA damage, were found to be significantly higher in the Fpg-modified comet assay than in the standard comet assay. Fpg-sensitive sites increased significantly in the septic rats (p<0.05) and RV significantly reduced Fpg-sensitive sites induced by sepsis in all tissue cells (35.2%, 37.6%, and 54.7% for TL, TI, and TM in the liver, respectively, and 61.1%, 54.7%, and 61.1% for TL, TI, TM in the kidney, respectively) (p<0.05).

DISCUSSION

Resveratrol treatment reduces the sepsis-induced oxidative stress, inflammation and DNA damage in the main target tissues in Wistar albino rats. Under normal physiological conditions, there is a balance between ROS formation and removal by endogenous antioxidant scavenging compounds. Oxidative stress can occur due to imbalance between the excessive production of ROS and the antioxidant defenses (22). The antioxidant defense system is impaired in sepsis (15). The reduction of GSH is supposed to reflect the intracellular oxidation impairing cellular functions and the strongest alteration in the antioxidant defense (23,24). The decreases in GSH and increases in MDA levels were determined in the hepatic and renal tissues of septic rats (25). In our study, as for our previous data (13-15), the increases in MDA levels and decreases in GSH levels and SOD and GPX activities in the liver and kidney were shown in the septic rats compared to the sham group.

An increasing protective and immunity response characterized by the increased nitric oxide (NO), ROS, and inflammatory cytokines such as TNF-alpha, cause sepsis progres-
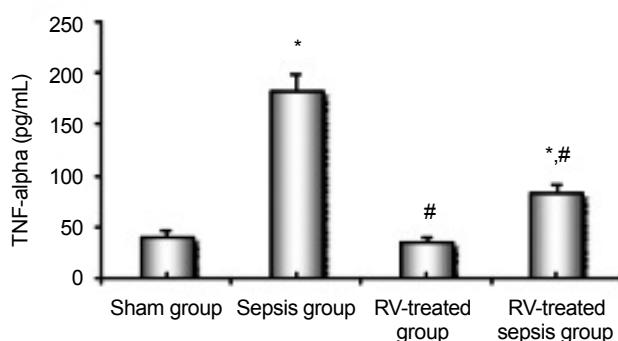
FIG. 1. Plasma TNF-alpha levels in the study group. TNF-alpha: tumor necrosis factor alpha; RV: resveratrol. *p<0.05, compared to sham group; #p<0.05, compared to sepsis group. The results were given as the mean ± standard deviation.

sion. TNF-alpha, a proinflammatory cytokine, manifests a considerably amplifying effect in the inflammatory response, and leads to severe tissue damages (1,2,15). The severity of tissue injury induced by sepsis is correlated with TNF-alpha level and there is a relation between inflammation and oxidative stress (26). In our study, as in our previous data (13-15), plasma TNF-alpha levels significantly increased in the septic rats, indicating an inflammatory response to sepsis.

Single and double DNA strand breaks, base modifications, fragmentation of deoxyribose, formation of DNA-protein cross-links as well as abasic sites may occur as a result of oxidative DNA damage. The alkaline single cell gel electrophoresis using Fpg, lesion-specific enzyme was used to detect oxidized purines showing the oxidative stress-induced DNA damage (8). In our study, Fpg-sensitive DNA lesions in the hepatic and renal cells significantly increased in the septic rats, referring to the oxidization of purines in the development of sepsis.

Resveratrol, a polyphenolic compound, is well known for its antioxidant feature. There is an increasing interest in RV, since RV may prevent or delay the ischemic and chemically-induced injuries, multi-organ dysfunctions, inflammations, infections, and heart disorders by inhibiting oxidation and the expression of inflammatory mediators and also by regulating microcirculation (3-6,9,27). Dalaklioglu et al. (28) found that RV treatment (20 mg/kg/day, ip) significantly prevented methotrexate-induced hepatotoxicity, as indicated by alanine transaminase, aspartate aminotransferase, and alkaline phosphatase levels and liver histopathology. RV significantly reduced the elevated levels of TBARS and activities of catalase and glutathione-S transferase in the hepatic tissue compared to the methotrexate-treated group. RV (5 mg/kg bw for 30 days) found a reduction of the levels of blood glucose, glycosylated hemoglobin, TNF-alpha, interleukin-1-beta, interleukin-6, NF-kappaB p65 unit and NO with increase in plasma
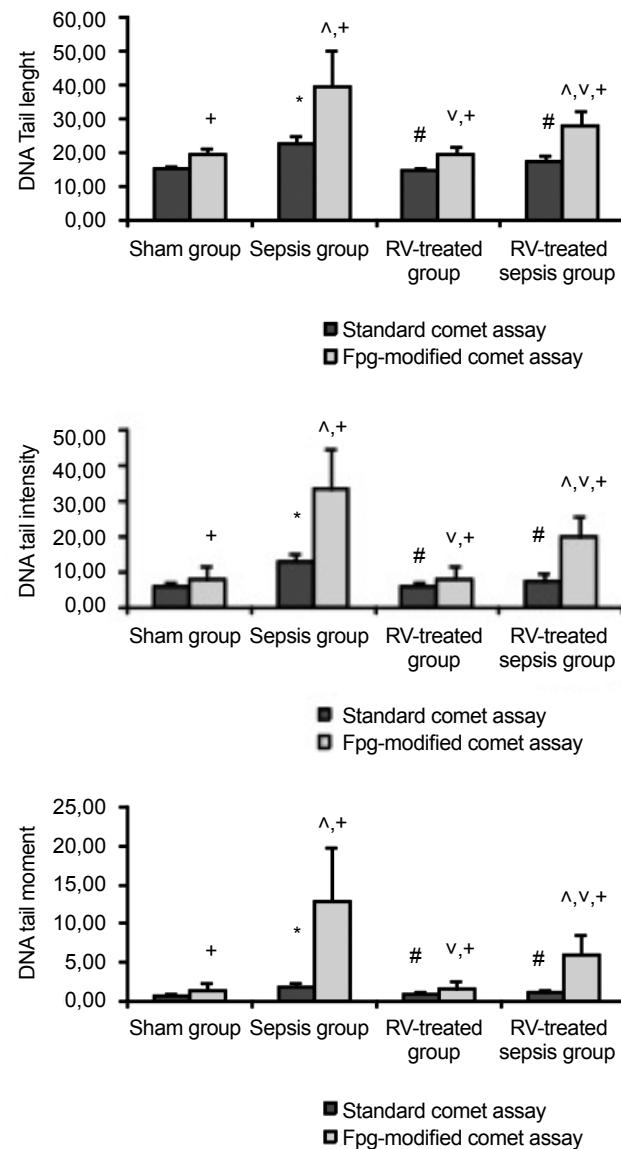

FIG. 2. a-c. DNA damage in the liver cells of the study groups. DNA damage was expressed as (a) DNA tail length; (b) DNA tail intensity; (c) DNA tail moment. RV: resveratrol. *p<0.05, compared to the sham group for the standard comet assay; #p<0.05, compared to the sepsis group for the standard comet assay; ^p<0.05, compared to the sham group for the Fpg-modified comet assay; ∨p<0.05, compared to the sepsis group for the Fpg-modified comet assay; +p<0.05, standard comet assay was compared to Fpg-modified comet assay. The results were given as the mean ± standard deviation.

insulin in diabetic rats (29). Bi et al. (30) found that RV treatment significantly inhibited the release of TNF-alpha and nitrite oxide induced by lipopolysaccharide (LPS) in rat cortical microglia and a mouse microglial cell line N9 *in vitro*. RV inhibited the degradation of IkappaB alpha, expression of iNOS and phosphorylation of p38 mitogen-activated protein kinases in N9 microglial cells treated with LPS. In sepsis progression, high-mobility group box 1 (HMGB1) protein, a nuclear pro-
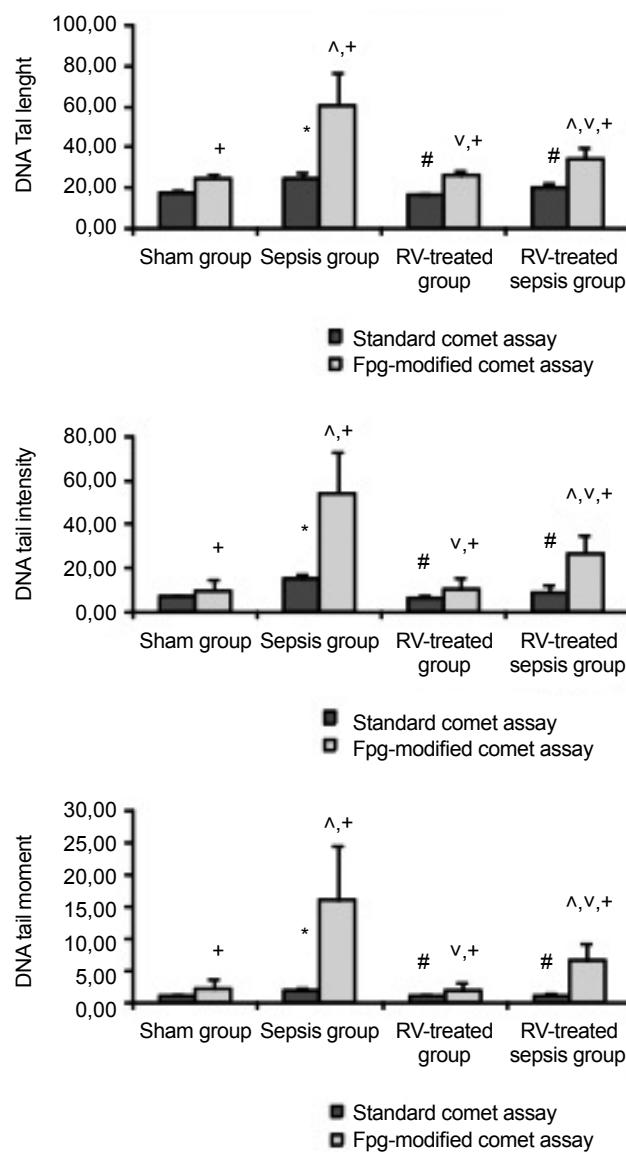
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FIG. 3. a-c. DNA damage in the kidney cells of the study groups. DNA damage was expressed as (a) DNA tail length; (b) DNA tail intensity; (c) DNA tail moment. RV: resveratrol. *p<0.05, compared to the sham group for the standard comet assay; #p<0.05, compared to the sepsis group for the standard comet assay; ^p<0.05, compared to the sham group for the Fpg-modified comet assay; ∨p<0.05, compared to the sepsis group for the Fpg-modified comet assay; +p<0.05, standard comet assay was compared to Fpg-modified comet assay. The results were given as the mean ± standard deviation.

tein binding to DNA molecule, promotes the activation of innate immune cells, which is suggested to be associated with liver injury. In liver cells, HMGB1 translocation was reported to be related to sirtuin 1 (SIRT1) upregulation. In the septic rats pretreated with RV (60 mg/kg/day for 3 days), serum aminotransferase activities, proinflammatory cytokines were reported to be reduced, thus liver histology was also improved. RV was suggested to prevent sepsis-induced liver damage

via SIRT 1-mediated HMGB1 (31). RV was found to modulate the expression of genes associated with proinflammatory cytokines by up-regulating the nuclear factor of kappa light chain gene enhancer in IkappaBalpha and down-regulating NFkappaBL1. RV increased the pro-inflammatory cytokines interleukin-6 and TNF-alpha in sepsis (32). Consistent with these studies, we observed that RV treatment significantly increased GSH levels and decreased MDA levels in the liver and kidney of septic rats. RV also significantly increased SOD and GPX activities in the liver and kidney of sepsis-induced rats. Plasma TNF-alpha level in the RV-treated septic rats was significantly lower than in the septic rats, indicating the protective effects of RV on tissue injury might be mediated by the suppression of the excessive inflammatory response.

In several models, it has been demonstrated that RV has anti-mutagenic and anti-carcinogenic activity (33). The chemopreventive effect of RV is possibly due to its ability to prevent DNA damage and to increase DNA repair (34-36). In the previous study with the peripheral lymphocytes of Wistar rats, RV (100 mg/kg bw, ip) was found to reduce the sepsis-induced oxidative DNA damage using the standard and formamidopyrimidine DNA glycosylase (Fpg)-modified comet assays (9).

In the study of Kolgazi et al. (37), RV was found to reduce sepsis-induced organ injury. RV was also found to induce premature senescence via decreasing SIRT1 and SIRT2 levels in human dermal fibroblasts. The decline in SIRT1/2 expression in response to RV treatment showed that DNA damage was regulated by RV (38). Kao et al. (39) reported that RV treatment reduced oxidative stress in human endothelial cells via induction of SIRT1 activation in endothelial cells. RV (100 mg/kg/day) was shown to protect radiation induced chromosome aberrations in mice (40). RV pretreatment (50 and 100 mg/kg/day, for 7 and 14 days) was found to reduce 8-OHdG, an oxidative DNA damage marker in urinary induced by cisplatin, via reducing oxidative DNA damage (41). Win et al. (42) showed that RV inhibited ROS-mediated strand breaks in Phi X-174 plasmid DNA. Consistent with these findings, we also found that RV treatment significantly decreased DNA damage induced by sepsis in the liver and kidney tissues of rats. The increases in DNA damage in the liver and kidney cells of RV treated-septic rats using the standard comet and Fpg-modified comet assay were much lower than in septic rats, showing that RV might prevent oxidative DNA strand breakages and increase DNA repair processes.

Our study has some limitations. The results of our study only give the protective effect of a single ip dose of RV (100 mg/kg) after 24 h of sepsis in Wistar albino rats. Due to the survival time of the septic rats being short, we only observed the rats for a short time after the induction of sepsis. We had some financial and technical limitations for this experiment.

More parameters such as DNA adduct formation, micronucleus formation and changes in gene expression related to DNA damage and inflammation would be investigated. Different experimental design at different treatment doses, route, and time and in different animal species would be performed. To determine the pretreatment effects of RV before the progression of sepsis, the rats would be pretreated with RV in the long-term before sepsis induction. The numbers of animals would be increased to achieve more accurate statistical results. The study would be repeated to show the reproducibility of our results. The level of RV and its metabolic products in biological samples would also be determined to reveal the dose-response relationship of RV.

In conclusion, DNA damage might be initiated in sepsis due to the induction of oxidative stress and it seems that RV via increasing the antioxidant status and DNA repair capacity *in vivo* has protective effects against the sepsis-induced oxidative stress and DNA damage. The uses of RV in clinical settings might be beneficial against the progression of tissue injuries induced by sepsis. It is suggested that RV treatment might reduce the oxidative DNA damage in sepsis-related multi-organ failures, however more investigations are required to clarify the act of protective mechanisms of RV against sepsis. We are planning to determine the pretreatment effects of RV at different oral doses for long-term before the progression of sepsis *in vivo*.

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