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Burn-induced distant organ injury in rats and the effect of minocycline

Sıçanlarda yanık ile uyarılan uzak organ hasarı ve minosiklinin etkisi

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ABSTRACT

Objectives: This study aimed to examine the anti-inflammatory potential of orally-administered minocycline, a semi-synthetic second-generation tetracycline, on burn-induced liver and kidney damage in rats.

Materials and Methods: Female Sprague-Dawley rats (250-300 g; n=7-8/group) were used. Burn and sham groups were exposed to 90 ^{0}C and 25 ^{0}C water bath for 10 s, respectively. Minocycline (20 mg/kg; twice daily; orogastrically) was administered for 24 h post-burn. After decapitation, trunk blood was collected for biochemical assays. Liver and kidneys were excised for histopathological evaluation and malondialdehyde (MDA), glutathione and chemiluminescence (CL) assays.

Results: Minocycline treatment did not exert a significant effect on serum alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, and creatinine levels of the burn group. Increased serum total oxidant status of the burn group was reversed ($P<0.001$) and liver microscopic lesion score showed a slight reduction ($P<0.01$) by minocycline. Minocycline did not cause a significant effect on increased tissue MDA and glutathione levels of the burn group but reversed the elevated luminol CL levels ($P<0.001$, for liver and $P<0.01$, for kidney, respectively).

Conclusion: Minocycline treatment to rats for 24 h post-burn was found to be effective to reduce oxidant production in the liver and kidney; however, it showed slight protection on these tissues against oxidant damage.

Keywords: Burn, Kidney, Liver, Minocycline, Rat

ÖZ

Amaç: Bu çalışma, oral uygulanan minosiklinin -yarı sentetik ikinci kuşak tetrasiklin– sıçanlarda yanık ile uyarılan karaciğer ve böbrek hasarı üzerine anti-inflamatuvar potansiyelini incelemeyi amaçlamıştır.

Gereç ve Yöntem: Çalışmada dişi Sprague-Dawley sıçanlar (250-300 g; n=7-8/grup) kullanıldı. Yanık ve taklit grupları, sırası ile 90 ^{0}C ve 25 ^{0}C'lik su banyosunda 10 saniye tutuldu. Minosiklin (20 mg/kg; günde iki kez; orogastrik yolla) yanık sonrası 24 saat süreyle uygulandı. Dekapitasyon sonrası, biyokimyasal ölçümler için gövde kanı toplandı. Histopatolojik inceleme ve malondialdehit (MDA), glutatyon ve kemiluminisans (CL) ölçümleri için karaciğer ve böbrekler çıkarıldı.

Bulgular: Minosiklin tedavisi yanık grubuna ait serum alanin aminotransferaz, aspartat aminotransferaz, kan üre azotu ve kreatinin düzeylerine anlamlı bir etki göstermedi. Yanık grubunda artmış serum toplam oksidan durum ($P<0.001$) minosiklin ile geri döndürüldü ($P<0.001$) ve karaciğer mikroskopik hasar skoru hafif azalma ($P<0.01$) gösterdi. Minosiklin yanık grubunda artmış doku MDA ve glutatyon düzeyleri üzerine anlamlı etki göstermedi ancak artmış luminol CL düzeylerini geri döndürdü ($P<0.001$, karaciğer ve $P<0.01$, böbrek).

Sonuç: Sıçanlara yanık sonrası 24 saat minosiklin tedavisi karaciğer ve böbrekte oksidan oluşumunu azaltmada etkin bulunmuştur; ancak bu dokuları oksidan hasara karşı hafif derecede koruyucu bir etki göstermiştir.

Anahtar kelimeler: Böbrek, Karaciğer, Minosiklin, Sıçan, Yanık

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Introduction

Severe burn injury causes an inflammatory response and serious metabolic disturbances such as cardiac dysfunction, acute respiratory distress syndrome, acute renal failure, and sepsis due to increased intestinal permeability. Multi-organ failure following a burn insult continues to be a serious problem in the clinical medicine.

Local burn insult produces damage in remote organs such as lung, liver, and kidneys within hours of injury. Thus, organ protection is essential for patients with severe burn/scald injuries. Although, the pathophysiological mechanism of injury in visceral organs distant to local burn is not clear, animal models and clinical trials have demonstrated that reactive oxygen species (ROS) play a critical role. Endotoxin and other bacterial-end products which become evident in the circulation as a result of burn wound colonization may activate macrophages and neutrophils. This leads to the release of oxidants, arachidonic acid metabolites, proteases, etc, which cause further injury in distant organs [1-6].

Minocycline is a semi-synthetic second-generation tetracycline with anti-apoptotic, immunosuppressive and anti-inflammatory actions in various pathological states including acne vulgaris, periodontitis, rheumatoid arthritis, asthma, scleroderma, Parkinson's disease and Huntington's disease [7-10]. Underlying mechanisms of minocycline's anti-inflammatory actions which are independent from its main clinical use as an antimicrobial, include the inhibition of ROS, and cytokine production, interference with protein synthesis and modulation of matrix metalloproteinase activity [11,12]. Previous studies have shown that minocycline inhibits phospholipase A2, and neutrophil migration and adhesion [13-15]. Additionally, minocycline directly scavenges ROS in several cell-free mixed-radical assays with a potency comparable to vitamin E [16]. Its oxidant-scavenging ability is attributed to its multiple-substituted phenol ring which generates a relatively stable and unreactive phenolic radical when binds to a free radical.

Chang et al. [17] established a unilateral partial-thickness second-degree burn-injury model in rats and examined the effect of minocycline on the level of pain. The results of the study demonstrated that daily treatment with minocycline, administered at the time of burn injury and for 7 days thereafter, attenuated ipsilateral and contralateral allodynia and significant suppression of evoked hyperexcitability of dorsal-horn neurons. However, the effect of minocycline on distant organ damage following a burn insult has not been investigated. Therefore, in the light of these previous observations, this study was undertaken to examine the effectiveness of minocycline in terms of its anti-inflammatory and anti-oxidant actions on the extent of damage the liver and kidney 24 h after a burn insult in rats.

Materials and Methods

Animals

Female Sprague-Dawley rats (250-300 g; n = 7-8 per group) were kept in a room at a constant temperature (22 ± 1 °C) with 12 h light/dark cycles and fed standard pellet chow and water *ad libitum*. The study protocol was approved by Marmara University Ethics Committee for the Care and Use of Experimental Animals. All experimental procedures were conducted in accordance with the Guide to the Care and Use of Laboratory Animals.

Experimental protocol

Under ether anesthesia, the dorsum of the rats were shaved and exposed to 90 °C water bath for 10 s (Burn group). This protocol has been shown to result in a partial-thickness second-degree skin burn involving 30% of the total body surface area [18]. The animals in sham group were dipped in a 25 °C water bath for 10 s. Minocycline (Carbosynth Ltd, Berkshire, UK) was dissolved in distilled water and administered orogastrically at a dose of 20 mg/kg (1 mL) twice daily 30 min after the burn insult. The treatment was continued for 24 h. The dose of minocycline was chosen according to literature data [19,20]. Sham group received the vehicle (distilled water; 1 mL) orogastrically for 24 h. Burn groups were resuscitated with lactated Ringer's solution (20 ml/kg, subcutaneously) immediately after the burn insult.

All animals were decapitated at 24 h after the burn insult. Blood samples were collected, and the liver and kidneys were harvested. Blood samples were centrifuged at 3,000 g for 15 min and serums were stored at -80 °C for the assessment of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine levels and for total oxidant status (TOS) and antioxidant capacity assays (TAC). Liver and kidney samples were taken for histological evaluation of the lesions by light microscopy or stored at -80 °C for subsequent measurement of malondialdehyde (MDA) level (a lipid peroxidation marker) and endogenous antioxidant glutathione content. Formation of ROS in tissue samples was monitored using the chemiluminescence (CL) method.

Measurement of serum ALT, AST, BUN and creatinine levels

Serum ALT, AST, BUN and creatinine levels were measured by means of standard spectrophotometric methods using

an autoanalyzer (Cobas 8000 Modular Analytics, Roche Diagnostics, Germany).

Serum TOS and TAC assays

Serum TOS and TAC levels were measured using the colorimetric methods using a commercial kit (Rel Assay Diagnostics, Gaziantep, Turkey) via an autoanalyzer (PG Instruments Ltd, Leicestershire, UK) as described previously [21,22]. TOC results are expressed as mmol H_2O_2 Equivalence per liter of serum and TAC results are expressed as mmol Trolox Equivalence per liter of serum.

Histological evaluation

For light microscopic investigation, liver and kidney samples were placed in 10% formaldehyde, dehydrated in ascending alcohol series (70%, 90%, 96% and 100%), and embedded in paraffin. For each animal, four randomly taken tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) and visualized with a microscope (Olympus BX51, Tokyo, Japan). Five to nine tissue sections from each animal were randomly chosen. From each section, five areas were randomly selected for histopathological examination. Semiquantitative scoring (maximum score=4) was performed using the criteria as demonstrated in Table I. The histopathologic score was determined by an experienced histologist (F.E) who was unaware of the treatment groups.

Measurement of MDA and glutathione levels

Liver and kidney samples were homogenized in 10 volumes of ice-cold 10% trichloracetic acid and centrifuged at 3,000 g for 15 min at 4 °C. Supernatant was removed and recentrifuged at 10,000 g at 4 °C for 8 min. Glutathione

was determined by a spectrophotometric method which is a modification of Ellman procedure [23]. Lipid peroxide levels are expressed in terms of MDA equivalents as nmol MDA per g tissue [24].

Chemiluminescence assay

Luminescence of the liver and kidney homogenates was recorded at room temperature using Mini Lumat LB 9506 luminometer (EG&G Berthold, Germany) in the presence of luminol or lucigenin 0.2 mM each. All counts were obtained at 15 s intervals for 5 min and the results were expressed as area under the curve (AUC) of relative light unit (rlu) for 5 min per mg of tissue. The calculation was based on the integration of the curve by the trapezoidal rule (a linear approximation) [25].

Statistical analysis

All data are expressed as means ± S.E.M. The histological data were compared using Mann-Whitney U non-parametric test. Other parameters were compared using two-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. Values of $P<0.05$ were regarded as significant. Calculations were done using Instat statistical analysis package (GraphPad Software, San Diego, CA, USA).

Results

Serum ALT, AST, BUN and creatinine levels

As demonstrated in Table II, serum ALT and AST levels – biomarkers of hepatocellular injury-showed marked increases in burn group compared to sham group ($P<0.001$).

Table I. Criteria used for microscopic scoring of burn-induced damage in liver and kidney samples.

Tissue	Score	Appearance
Liver	0	Normal
	1	Expansion and vacuolization of hepatocytes
	2	Vascular congestion and expansion of sinusoids
	3	Mild increase in number of Kupffer cells
	4	Severe increase in number of Kupffer cells
Kidney	0	Normal
	1	Degeneration of proximal tubuli
	2	Vascular congestion
	3	Mild glomerular congestion and degeneration
	4	Severe degeneration of glomerular structure and expansion of Bowman's space

Table II. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine levels of the experimental groups.

	ALT (U/L)	AST (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
Sham	51.50 ± 3.27	167.40 ± 15.38	12.94 ± 0.63	0.25 ± 0.001
Burn	$124.00 \pm 4.42^{***}$	$616.00 \pm 38.29^{***}$	$25.08 \pm 3.26^{**}$	$0.30 \pm 0.02^{*}$
Burn+Min	$127.80 \pm 6.61^{***}$	$527.30 \pm 49.58^{***}$	$17.93 \pm 2.04^{*}$	$0.31 \pm 0.02^{*}$

Min: minocycline
Data are means ± SEM of 8 rats for each group.*$P<0.05$, **$P<0.01$, and ***$P<0.001$, compared to sham group.

In minocycline-treated burn group, the serum ALT and AST levels did not show statistically significant differences when compared with those of the untreated burn group. Burn insult also caused elevations in serum BUN and creatinine levels indicating kidney dysfunction ($P<0.01$, for BUN and $P<0.05$, for creatinine). Similarly, minocycline treatment had no significant effect on these parameters (Table II).

Serum TOS and TAC assays

As shown in Table III, TOS level of the burn group was significantly increased relative to that of the sham group ($P<0.001$). Minocycline treatment to burn animals was associated with decreased TOS levels in comparison to untreated burn group ($P<0.01$). Although, the sham and burn groups did not differ in terms of TAC levels, minocycline treatment increased the TAC levels of the burn animals in comparison to sham group ($P<0.05$).

Histological evaluation

Liver samples of the burn group revealed sinusoidal congestion and dilation, inflammatory cell infiltration, increased number of activated Kupffer cells and perinuclear vacuolization and edema of the hepatocytes (Figure 1). The extent of sinusoidal

dilation and congestion and the number of degenerated hepatocytes seemed to decrease in minocycline-treated burn group. The histopathological changes observed in liver samples were in the same line with the microscopic lesion scores of the experimental groups. That is, the microscopic lesion score of the burn group (3.44 ± 0.22; $P<0.001$) showed a slight reduction by minocycline treatment (2.44 ± 0.17; $P<0.01$) (Figure 2).

In burn group, the kidney samples revealed glomerular and vascular congestion, expansion of Bowman's space, degeneration and desquamation of tubular cells and expansion of the tubuli (Figure 3). Although minocycline decreased glomerular congestion it did not modulate the severity of the damage in terms of other microsocopic scoring criteria. The kidney microscopic lesion scores of the burn and minocycline-treated burn groups were 3.50 ± 0.19 and 2.81 ± 0.26, respectively (Figure 2).

Tissue MDA and Glutathione levels

The burn group presented increased MDA and glutathione levels in both liver and kidney samples when compared with the sham group. Burn-induced changes of these parameters were preserved by minocycline treatment (Table IV).

Table III. Serum total oxidant status (TOS) and total antioxidant capacity (TAC) levels of the experimental groups.

	TOS (mmol H2O2 Equiv/L)	TAC (mmol Trolox Equiv/L)
Sham	7.14 ± 1.33	4.13 ± 0.27
Burn	$27.43 \pm 4.00^{***}$	4.61 ± 0.13
Burn+Min	$8.98 \pm 1.04^{++}$	$5.04 \pm 0.23^{*}$

Min, minocycline
Data are means ± SEM of 8 rats for each group. *$P<0.05$, and ***$P<0.001$, compared to sham group.++$P<0.01$, compared to burn group.
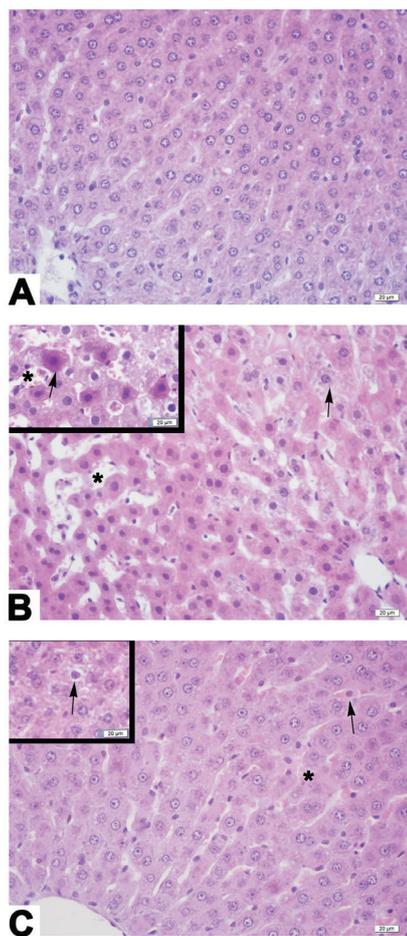
Figure 1. Micrographs illustrating the histological appearances of liver samples from experimental groups. Sham group (A) with normal liver parenchyma. Burn group (B), with sinusoidal expansion and congestion (*), degeneration of hepatocytes with vacuolization (arrow) or eosinophilic cytoplasm (arrow, inset). Burn+minocycline group (C) with less sinusoidal expansion and congestion (*) and less number of degenerated hepatocytes (arrow). H&E staining, original magnifications 200x, inset 400x.
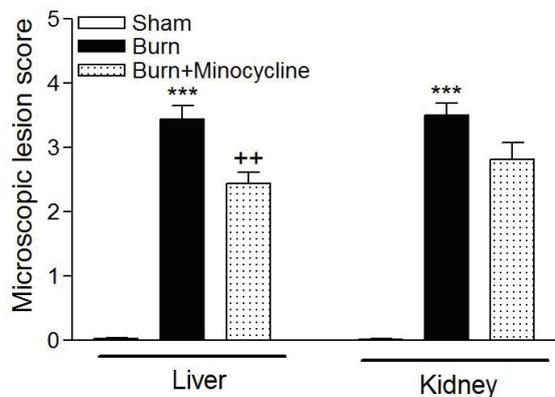

Figure 2. Microscopic lesion score in liver and kidney samples of the experimental groups. ***$P<0.001$, compared to sham group.$^{++}P<0.01$, compared to burn group.
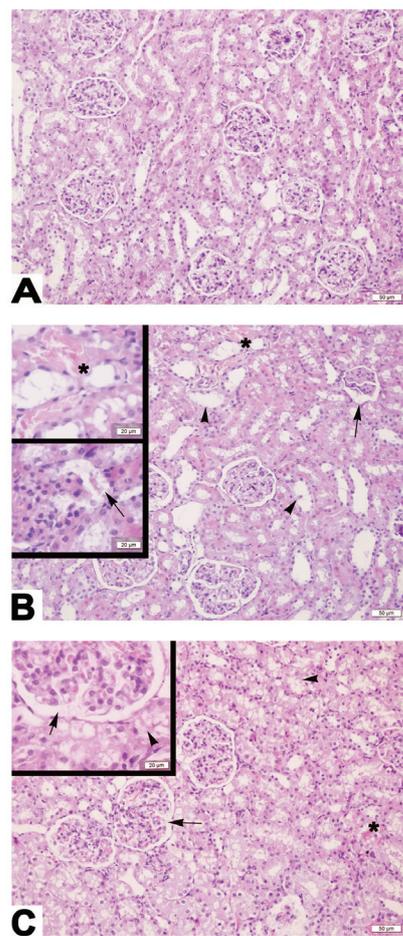

Figure 3. Micrographs illustrating the histological appearances of kidney samples from experimental groups. Sham group (A) with normal renal parenchyma. Burn group (B), with glomerular congestion, expansion of Bowman's space and cell debris (arrow), vascular congestion (*), and expansion of tubuli and tubular degeneration (arrow head). Burn+minocycline group (C) with less glomerular congestion, Bowman's space with normal morphology (arrow), expansion and degeneration in less number of tubuli (arrow head). H&E staining, original magnifications 200x, inset 400x.

Chemiluminescence values

Both liver and kidney luminol-enhanced CL levels showed significant increases in the burn group compared to sham ($P<0.01$). The elevations of luminol-enhanced CL levels in both tissues were reversed by minocycline ($P<0.001$, for liver and $P<0.01$, for kidney). No statistically significant changes were observed among the experimental groups in terms of lucigenin-enhanced CL levels (Table V).

Table IV. Liver and kidney malondialdehyde (MDA) and glutathione levels of the experimental groups.

	Liver		Kidney	
	MDA (nmol/g)	Glutathione (μmol/g)	MDA (nmol/g)	Glutathione (μmol/g)
Sham	9.63 ± 1.01	3.68 ± 0.14	13.89 ± 0.68	1.39 ± 0.16
Burn	25.42 ± 1.44***	4.42 ± 0.21*	18.82 ± 0.78***	2.92 ± 0.26***
Burn+Min	22.18 ± 3.17*	4.49 ± 0.12***	26.02 ± 1.92***++	3.59 ± 0.18***

Min: minocycline
Data are means ± SEM of 8 rats for each group.*$P<0.05$, ***$P<0.001$, compared to sham group.++$P<0.01$, compared to burn group.

Table V. Liver and kidney luminol- and lucigenin-enhanced chemiluminescence (CL) levels of the experimental groups.

	Liver		Kidney	
	Luminol-enhanced CL (rlu/mg)	Lucigenin-enhanced CL (rlu/mg)	Luminol-enhanced CL (rlu/mg)	Lucigenin-enhanced CL (rlu/mg)
Sham	1.94 ± 0.19	2.59 ± 0.15	1.55 ± 1.13	1.75 ± 0.08
Burn	3.51 ± 0.36**	3.05 ± 0.25	2.78 ± 0.35**	1.87 ± 0.17
Burn+Min	1.35 ± 0.16+++	2.79 ± 0.44	1.73 ± 0.25++	1.93 ± 0.11

Min: minocycline
Data are means ± SEM of 8 rats for each group. **$P<0.01$, compared to sham group.++$P<0.01$, and +++$P<0.001$, compared to burn group.

Discussion

Severe burn may lead to multiple organ dysfunction and death as a result of disruption of the body's homeostatic balance. This study examined the histopathological and biochemical changes that occur in liver and kidneys of rats 24 h after induction of partial-thickness second-degree skin burn and evaluated the effectiveness of minocycline to limit the extent of tissue injury via its putative anti-inflammatory and anti-oxidant actions.

The relationship between local inflammation and subsequent systemic inflammatory response is unclear. In addition to local inflammation, severe skin burn induces the systemic inflammatory response syndrome (SIRS) [26]. The mechanisms that are responsible for distant organ damage after local injury, include the systemic liberation of proinflammatory mediators, neutrophil attraction, and activation of the sympathetic inflammatory signaling [27,28]. It is known that gut barrier function is compromised following thermal injury and that endotoxins and other bacterial by-products as a result of burn wound colonization contribute to damage in distant organs. Endotoxin translocates across the gastrointestinal tract barrier within 1 h of thermal

injury and plasma endotoxin concentration reaches a peak at 12 h and 4 days postburn [29,30]. Endotoxins are potent activators of the macrophages and neutrophils. Activation of these inflammatory cells leads to the release of oxidants, arachidonic acid metabolites and proteases, all of which cause further local and systemic inflammation following burn [31]. In our study, we observed severe tissue damage in both liver and kidney samples at microscopic level and increased blood ALT, AST, BUN and creatinine levels in the burn group compared to sham implying hepatic and renal dysfunction. There were increased lipid peroxidation (as shown by MDA assay) and antioxidant glutathione levels in the liver and kidney samples of burned animals when compared with those of the sham group. We also observed marked elevations in luminol-enhanced CL values in the liver and kidneys and increase in TOS values in the serum of burned animals. All these results suggest that partial-thickness second-degree skin burn rat model causes oxidative stress in liver and kidney at 24 h post-burn.

Tetracycline antibiotics were the first major class of 'broad-spectrum antibiotics' which have been used since 1940s against a wide range of both Gram-positive and

Gram-negative pathogens, mycoplasmas, intracellular chlamydia, rickettsia and protozoan parasites. The second generation of semisynthetic tetracyclines, minocycline and doxycycline, with improved antimicrobial potency, were introduced during the 1960s. Tetracyclines, in addition to their antimicrobial properties, exhibit inhibitory activity toward the inflammatory cascade and mediators of tissue damage [32]. As reported, minocycline also possesses potent anti-apoptotic properties. It inhibits caspase 1 and 3 expressions and directly blocks cytochrome c release from mitochondria [10,33]. Recent studies have shown that anti-inflammatory and immunomodulatory actions of minocycline are not related to its antimicrobial property [34]. Minocycline suppresses the secretion interleukin (IL)-1beta, IL-8 and tumor necrosis factor (TNF)-alpha, in macrophages stimulated by lipopolysaccharides (LPS) [35]. An *in vitro* study by Leite et al. [20] tested the antioxidant activity of minocycline by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and found that minocycline has an antioxidant activity which is more potent than alpha-tocopherol. In the same study, the anti-inflammatory properties of doxycycline and minocycline were also compared in *in vivo* and *in vitro* settings. In general, the anti-inflammatory activity of doxycycline was found to be higher as compared to that of minocycline, although the radical scavanging activity of minocycline was of a magnitude 10 times higher [20]. A very recent *in vitro* study by Parenti et al. [36] demonstrated that minocycline significantly inhibited oxidative burst of human polymorphonuclear (PMN) cells stimulated with N-formylmethionyl-leucyl-phenylalanine (FMLP) in a concentration-dependent manner and impaired PMN transendothelial migration. Data from *in vitro* rat brain homogenate lipid peroxidation and DPPH assays showed that minocycline had an IC_{50} of 3-40 mM and was 9-250 times more potent as a scavanger than doxycycline [37]. ROS scavenging ability of minocycline is likely to be attributed to its unique structure, with diethylamino group on the phenolic carbon that provides steric hindrance [37]. Moreover, minocycline and to a lesser extent doxycycline inhibits phospholipase A2, neutrophil migration and adhesion, and lymphocyte proliferation [38-40]. Overall, it is believed that the anti-inflammatory action of minocycline involves the inhibition of mediators of the inflammatory process, including interleukins, prostaglandins, inducible nitric oxide synthase (iNOS), and an antioxidant activity.

ROS can cause tissue damage if the rate of their production exceeds the capacity of the endogenous antioxidant enzymes. Increased oxidative stress and impairment of antioxidant defenses by the deleterious effect of ROS contribute to the pathogenesis of the inflammatory conditions. CL measures light production as a byproduct of oxidative reactions and is a commonly used technique to estimate ROS generation in various tissues. Luminol and lucigenin CL probes have different selectivity towards radicals. Luminol quantifies hydrogen peroxide, hydroxyl radical, hypochlorite, peroxynitrite, and lipid peroxyl radicals whereas lucigenin is selective for superoxide radical [41]. In our study, we observed elevated luminol CL levels in liver and kidney samples of burn animals indicating the increased generation of hydrogen peroxide, hydroxyl radical, hypochlorite, peroxynitrite, and lipid peroxyl radicals in these tissues post-burn. In our study, minocycline at a daily dose of 40 mg/kg (20 mg/kg, twice daily) did not exert a statistically significant effect on the levels of lipid peroxidation and glutathione in liver and kidney samples of burn animals. However, there were marked reductions in luminol-enhanced CL levels in both tissues and the total oxidant status (TOS) in blood by minocycline treatment. Thus, these findings may suggest that minocycline is effective to reduce the level of ROS production in liver and kidney following burn. Another finding of the study was that minocycline did not attanuate the increased serum levels of liver enzyme activities and BUN and creatinine as indicators of cellular dysfunction. Similarly, the extent of liver and kidney injury at microscopic level in burn group showed slight preservation by minocycline. Thus, although minocycline treatment to burn animals suppresses oxidant production in liver and kidney it is not effective to protect these tissues against damage. To reveal minocycline's effectiveness on the extent of burn-induced tissue damage, a burn protocol possibly with prolonged minocycline treatment is warranted.

In conclusion, the administration of minocycline for 24 h beginning at the time of burn injury showed slight protection on liver and kidney against oxidant stress and damage.

Conflict of interest

None declared.

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