Introduction

Thermal burns and related injuries are a major cause of death and disability, especially in subjects under the age of 40. Even in developed countries, more than 2 million individuals annually are burned seriously and require medical treatment [1]. The average burn patient is 24.4 years old and has a mean burn size of 19% of the total body surface area (TBSA) [2].

The local and systemic inflammatory response to thermal injury is extremely complex, resulting in both local burn tissue damage and deleterious systemic effects on all other organ systems distant from the burn area itself. Although the inflammation is initiated almost immediately after the burn injury, the systemic response progresses with time, usually peaking 5 to 7 days after the burn injury [3-5]. Much of the local and certainly the majority of the distant changes are caused by inflammatory mediators [6-8]. Thermal injury initiates systemic inflammatory reactions producing burn toxins and oxygen radicals and finally leads to peroxidation. The relationship between the amount of products of oxidative metabolism and natural scavengers of free radicals determines the outcome of local and distant tissue damage and further organ failure in burn injuries [9]. The injured tissue initiates an inflammation-induced hyperdynamic, hypermetabolic state that can lead to severe progres-
Trimetazidine for burn-induced intestinal mucosal and kidney injury

Trimetazidine (TMZ) has been used in cardiology practice for protection from ischemia-reperfusion injury. But its effects on intestinal mucosa are not well known. The aim of this experimental study was to investigate the protective effect on intestinal mucosa of TMZ and its relation with free oxygen radicals in intestinal mucosal injury model due to thermal injury in rats [12-14]. And we also aimed to show the effect of Trimetazidine (TMZ) on the kidney damage originated from distant skin thermal injury. TMZ, anti-oxidative agent is in use for the treatment of coronary heart disease and has been the subject of several investigations since 1970s. It has been shown that TMZ had a cytoprotective effect without impairing hemodynamics; yet, exact mechanism of TMZ is unknown. Different pathophysiological changes have been shown in kidney; tubular changes, glomerular changes, venous congestion and massive tissue edema have been come across.

Materials and methods

Experimental protocol

A total of 30 Sprague-Dawley type male rats (200-250 gr) used in the first group to show the effect of TMZ on burn-induced intestinal mucosal injury. 24 Sprague-Dawley female rats weighing 230 grams were used in the second group to show the effect of TMZ on oxidative kidney damage. After anesthesia induction back regions of all animals were shaved. Both groups were divided into 3 groups; test groups (thermal injured and TMZ injected; Ia and IIa), control groups (thermal injury was performed; Ib, IIb) and sham-control groups (Ic and IIc). Rats in I-IIa and I-IIb groups were taken into water of 99°C about 10 seconds in order to perform thermal injury on back which was approximately 30-35% of the whole body surface. The third groups Ic and IIc were sham-control groups. In groups Ib and IIb (burned control group) (n=13), after thermal injury, 2 mL of normal saline were injected intraperitoneally. In groups Ia and IIa (TMZ group) (n=12), after thermal injury, 3 mg/kg TMZ (TMZ, Vasterel; Servier, Gidy, France) were injected intraperitoneally in 2 mL of normal saline. In Group Ic (Sham control group (n=5) are taken into water that is 21°C, 2 mL of normal saline were injected intraperitoneally without thermal injury. All rats were sacrificed 5 hours after the burn injury by 50 mg/kg ketamine hydrochloride (Ketalar: Darke, Dawis, Eczacibasi, Turkey) and 10 mg/kg xylazine hydrochloride (Rompun: Bayer, Leverkusen, Germany). Nephrectomy was done in the second group and the kidneys were left in 10 % buffered formalin overnight. Later, stereological, histopathological examinations and malondialdehyde determination were completed. Ethical approval for this research was received from the local ethical committee.

Measurements

Concentrations of lipid peroxidation products, including malondialdehyde (MDA), in intestinal tissue were determined. The level of glutathione (GSH), an antioxidant substrate in intestinal tissue, was also measured malondialdehyde content was measured by the method of Tani-zawa et al [16]. In brief, 5 cm intestinal segments were homogenized with a Teflon-glass homogenizer in 12 ml saline solution containing EDTA 0.003 mol/L. The homogenized tissue was centrifuged at 3000 rpm for 10 minutes, and 200 ml of supernatant was added to a reaction mixture consisting of 1.5 ml of 0.8% thio-barbituric acid and 1.5 ml of acetic acid buffer 2.0 mol/L (pH: 3.6). This solution was placed in a 95°C water bath for 15 minutes. After addition of 5 ml n-butanol/pyridine mixture, samples were agitated and subsequently centrifuged at 3000 rpm for 15 minutes. The fluorescence intensity of the upper organic layer was measured at excitation and emission wavelengths of 515 and 553 nm, respectively, by a spectrofluorometer (model FP-77; Japan Spectroscopic Co., Tokyo, Japan). MDA bis (diethylacetal) (Sigma) was used as the standard. Intestinal protein content was determined by the method of Lowry et al. Glutathione was determined by a commercially available test kit (Bioxytech GSH-400; OXIS International, Portland, OR, USA). Tissue MDA, MPO and GSH levels were measured [15-17].

All experimental data are expressed as the mean and SD. The significance of difference among all groups was analyzed using one-way ANOVA.

Histopathology

After tissue processing in different concentrations of ethyl alcohol and xylene, kidneys were...
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Table 1. Intestinal MDA levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>MDA</th>
<th>P values</th>
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<tbody>
<tr>
<td>Ib</td>
<td>Burned control group (n=13)</td>
<td>17 ± 9.1</td>
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<tr>
<td>Ia</td>
<td>TMZ group (n=12)</td>
<td>14 ± 4.8</td>
<td>0.5</td>
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<tr>
<td>Ic</td>
<td>Sham control group (n=5)</td>
<td>12.4 ± 1.2</td>
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Table 2. Intestinal GSH/ GSSG ratios

<table>
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<th>Group</th>
<th>Description</th>
<th>GSH</th>
<th>P values</th>
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<tr>
<td>Ib</td>
<td>Burned control group (n=13)</td>
<td>29.6 ± 5.1</td>
<td></td>
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<tr>
<td>Ia</td>
<td>TMZ group (n=12)</td>
<td>29.2 ± 3.7</td>
<td>0.234</td>
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<tr>
<td>Ic</td>
<td>Sham control group (n=5)</td>
<td>26.6 ± 3.3</td>
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Table 3. Intestinal MPO levels

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<tr>
<th>Group</th>
<th>Description</th>
<th>MPO</th>
<th>P values</th>
</tr>
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<tbody>
<tr>
<td>Ib</td>
<td>Burned control group (n=13)</td>
<td>31.3 ± 6</td>
<td></td>
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<tr>
<td>Ia</td>
<td>TMZ group (n=12)</td>
<td>25.1 ± 8.7</td>
<td>0.050</td>
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<tr>
<td>Ic</td>
<td>Sham control group (n=5)</td>
<td>24.6 ± 3.1</td>
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Histopathological examinations were performed on the same sections.

In addition to histological evaluation of the tissue, qualitative study of Klausner et al. type [11], acute tubular necrosis (ATN) was evaluated by a quantitative grading scale (0 - 4). Grade 0: Normal Kidney; Grade 1: minimal ATN< 5%; grade 2: mild ATN, 5 - 25%; grade 3: moderate ATN, 25 - 75%; grade 4: severe ATN, > 75%.

Kidney volume estimations

In biomedicine, organ volumes can be estimated unbiased, using the Cavalieri principle. This basic technique was demonstrated 300 years ago by the Italian mathematician Cavalieri in order to obtain volume estimations. According to this principle, the volume of an object could be estimated from a set of parallel, known distance two-dimensional slices randomly cut through the object.

Cavalieri’s volume estimation was used to estimate the kidney volumes as an unbiased indicator of tissue edema. In this principle, the equally thick uniform random sections were cut exhaustively throughout the organ. The sections mounted on the slides and covered by cover slips were then laid down, with the same sides of each section uppermost, and then overlaid with a uniform random quadratic grid of points. The total number of points (Sp) falling on the kidney sections was recorded. Each point had an area domain (a/p) and the mean thickness of the slices (T), was known. The volume of kidneys was calculated by multiplying the total number of points by the area of each point and the mean thickness [12]. The formula used in this calculation was given below.

\[\text{Volume Estimation} = T \times \frac{a}{p} \times SP\]

T: Section thickness; a/p: Area per point; SP: Total number of points counted throughout the kidney

In our study, each kidney were cut into 10 - 15 sections for volume estimation on the sagittal plane and on average 150 - 200 points were counted by a square grid test system (a/p: 0.25 mm²) with the help of a lens. Point counting and volume estimations were repeated three times and the means were obtained.

Results

Results of the study are summarized in Tables 1, 2, 3 and 4. Malondialdehyde (MDA) levels in groups as an indicator of oxidative damage were analyzed by Kruskal-Wallis and Mann-Whitney U tests. Although intestinal MDA levels in TMZ group (Group Ib) was lower than the
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There was no statistical significance among groups (Table 1). There was no statistical difference in intestinal MDA levels when compared to TMZ group with burned control group (p=0.4), or TMZ group with sham control group (p=0.4), or sham control group with burned control group (p=0.5). Also there was no statistical significance in intestinal GSH/GSSG ratios among groups (Table 2). There was no statistical difference in intestinal GSH/GSSG ratios between TMZ group and burned control group (p=0.7) or TMZ group and sham control group (p=0.81), or sham control group and burned control group (p=0.3). There was a statistical difference in intestinal MPO levels among groups (p<0.05) (Table 3). Intestinal MPO levels was significantly lower in TMZ group than burned control group (p=0.044), but not than sham control group (p=0.641). Also there was a statistical difference between sham control group and burned control group (p=0.022).

In the renal injury tested groups, TMZ group showed lower MDA levels than normal while the Group IIa showed significantly higher levels (p<0.05). All the data were showed in the Table 4.

Histopathological evaluation

In burn group, most of the glomeruli have degenerative changes. The picnotic nuclei were observed in the glomeruli (Figure 1A). There were hemorrhagic foci in glomeruli cells (Figure 1B). Glomerular spaces were enlarged in moderate to severely degenerative glomeruli and some of the parietal layer epithelium cells were disappeared (Figure 1C). Distal tubuli had normal appearance while distal their epithelium cells had vacuolar changes in their cytoplasmic space and proximal tubuli epithelium have lost their cubic structure and have become flattened and their nuclei have become heterochromatic (Figure 1D). Another important observation in this group was venous congestion.

In TMZ group, the appearance of glomeruli were comparable with the control group, however, glomeruli cells were not picnotic and the enlargement in the glomeruli spaces were not as large as in the burn group (Figure 1D, 1E). Proximal tubuli epithelium was normal and no pathology in tubuli was observed (Figure 1F).

In control group, normal histology was observed (data not shown).

Volume estimation results

Cavalieri’s volume estimation results are shown in Figure 2. In the calculation of Cavalieri estimation, coefficient of error (CE) was obtained to evaluate the reliability of the point density on the grids and organ section intervals. Stereologists use different formulas to calculate the CE. The volumes of kidneys estimated by Cavalieri principle and the estimates of three repetitions correlated well with each other. The range of CE values changed from 1.9 to 2.7%, which are reasonable values for the volume estimates.

Discussion

A dynamic ileus, gastric dilatation, increased gastric secretion and ulcer incidence, gastrointestinal hemorrhage and local and general distribution of the blood flow with a decrease of mesenteric blood flow are among the effects of thermal injury on the gastrointestinal system [18]. A decrease in mesenteric blood flow has been described in a number of burn and smoke inhalation animal models, even in the absence of any evidence of inadequate systemic perfusion [19]. The effect of acute burn trauma, produced by hot water scalding in the rat, has demonstrated that there is decreased nutrient absorption (glucose, calcium and amino acids) and DNA synthesis in the small intestine [20]. Intestinal ischemia resulting from decreased splanchnic blood flow may activate the neutrophils and tissue-bound enzymes such as xanthine oxidase and these factors destroy the gut mucosal barrier and result in bacterial translocation. These data indicate an early post burn

<table>
<thead>
<tr>
<th>Table 4. Kidney Tissue MDA levels</th>
<th>MDA</th>
<th>P values</th>
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<tbody>
<tr>
<td>Group IIb Burned control group (n=8)</td>
<td>0.12 ± 0.17</td>
<td></td>
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<tr>
<td>Group IIa TMZ group (n=8)</td>
<td>0.24 ± 0.20</td>
<td>0.01</td>
</tr>
<tr>
<td>Group IIc Sham control group (n=8)</td>
<td>0.6 ± 0.27</td>
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Gut barrier leak after the burn, which may be the source of circulating endotoxin [21]. Endotoxins are potent activators of the macrophages and neutrophils. This leads to the release of massive amounts of oxidants, arachidonic acid metabolites and proteases, which cause further local and systemic inflammation in burn-induced tissue damage [22].

Ischemia-Reperfusion Injury (I/R) is the prime culprit in many clinical entities. It is the injury sustained after an ischemic event and subsequent restoration of blood flow that far surpasses that expected by ischemia alone [23-25]. Small intestine is the only organ to absorb nutrients through enteral feeding. It was noted that the small intestine is disproportionately susceptible to ischemia during circulatory shock [26]. Ischemia activates xanthine oxidase, which then generates a massive burst of oxygen and superoxide free radicals when oxygen is reintroduced at reperfusion [27]. These radicals and their toxic oxygen metabolites, by direct action and by secondary activation of circulating neutrophils, generate much of the mucosal injury that had been previously attributed to anoxia itself. The ischemia-reperfusion (I/R) of small intestine can also cause morphologic changes [28] and increase the mucosal permeability that impairs the digestive and absorptive function of the small intestine.

Figure 1. Pathological sections from renal tissue stained by Hematoxylin – Eosin (H.E). Upper line sections show the samples from group IIb and below line from group IIa. A - B) A section from group IIb; pathological glomerulus (H.E. X 40 and X 100). C) A section from group IIb; proximal and distal tubuli in renal cortes (H.E., X 40). D - E) A renal tissue section from group IIa; glomerulus and glomerular space (H.E, X 40 and X 100). F) A section from group IIa; glomerulus, glomerular space, proximal and distal tubule (H.E., X 10). Abbreviations used: Glomerulus (G), glomerular space (GS), proximal tubule (PT) and distal tubule (DT).

Figure 2. Volume estimation results of the kidney.
Reperfusion of ischemic organs can result in tissue injury that manifests as micro vascular and parenchymal cell dysfunction. This observation suggests that some reactions are initiated by the return of oxygenated blood to the ischemic tissue. Reperfusion of ischemic tissues produces injury led to the concept that reperfusion injury may be mediated, at least in part, by the formation of reactive oxygen metabolites, free radicals including superoxide (O2-) and the hydroxyl radical (OH-) [29]. Peroxidation of membrane lipids can disrupt membrane fluidity and cell compartmentalization, which can result in cell lysis. Studies have demonstrated that free radicals are formed immediately after reperfusion of the ischemic intestine [29, 30]. These free radicals, which derived from the xanthine oxidase reaction, induce the production of neutrophils and injure mucosal cells of the small intestine during I/R process [30].

The ability of free radicals to initiate lipid peroxidation can result in the formation of lipid-derived radicals and degradation of products MDA. Concentrations of tissue MDA in small intestine proved to be elevated after I/R injury [17, 18]. GSH is an important antioxidant substrate that can be utilized to absorb free radicals that emerge during I/R injury. Changes in tissue GSH contents can be used to detect the variation of free radicals and thus I/R-induced damage. Our results show that TMZ decreased MPO levels, but no effect on GSH/GSSG and MDA levels.

TMZ has a protective effect on gastric mucosal due to thermal injury in rats. In conclusion TMZ decreases MDA and MPO levels, but no effect on GSH/GSSG levels [33, 34].

The major effects of TMZ are that it prevents the intracellular decrease of ATP levels, provides intracellular decrease of inorganic phosphate deposits, intracellular acidosis, decrease of mitochondrial oxidative damage so that TMZ shows a protective effect against oxidative damage, prevents free radical forming due to this damage and adverse effects of these radicals on the cellular membrane and prevents accumulation of neutrophils. Free oxygen radical production during shock, vascular surgery and renal transplantation is the most important mechanism in tissue injury. Data supporting this hypothesis are the alteration of lipid peroxidation product levels, the decrease in endogenous anti-oxidants and glutathione. In different studies performed on kidneys, anti-ischemic effect of TMZ was shown at cellular level [35-37]. In our study, it is shown at the level of tissue by unbiased stereological tools, histopathological evaluation and MDA levels in a longer duration of ischemia.

In terms of levels of MDA, a significant difference was shown between the TMZ and sham groups. Lipid radicals can be produced either by radiation or several radical reactions. Lipid radical causes cellular damage by the peroxidation of unsaturated fat acids existing on the cellular membranes. Lipid radical interacts with oxygen and forms Lipid peroxyl-radical. All biological membranes are susceptible to lipid peroxidation. Starting from the alteration of membrane permeability, it might cause structural and functional membrane impairments and finally membrane integrity might be impaired. Lipid peroxyl-radical forms other “Lipid hydroperoxydes” which in turn form highly toxic radicals the most toxic of which are “aldehydes” [38].

Conclusion

TMZ decreased MPO levels, but no effect on GSH/GSSG and MDA levels. MPO levels were significantly lower in TMZ group than burned-control group (p<0.05).

In this study, TMZ seems to be a protective on intestinal mucosa due to thermal injury in rats.

In addition, the results indicated that TMZ attenuated kidney damage originated from severe thermal injury of the skin in this animal model. Further studies, in terms of unbiased neutrophil number estimation as another index of oxidative injury, would be useful to further support these results.

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