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Effect of melatonin on the gastric antioxidant defence in experimental burn trauma

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Abstract: Severe burn trauma triggers oxidative gastric mucosal injury. The purpose of this study was to investigate the antioxidant defence mechanisms and protective effect of melatonin in the gastric mucosa after burn injury. In order to investigate the mechanisms involved in the gastric antioxidant defence in a rat burn model, quantitative real-time PCR and immunohistochemistry techniques were applied. An analysis of glutathione peroxidase 4 (GPx4), glutathione reductase (GR), and catalase (Cat) gene expression was performed along with the evaluation of the gastric Cu/Zn superoxide dismutase (Cu/Zn SOD) activity. Melatonin was applied immediately and 12 h after 30% of total body surface area burns. The burn injury significantly increased the *Gpx4* mRNA ($P < 0.000\ 1$) and *Gsr* mRNA ($P < 0.000\ 1$) expression. It also had a slight positive effect on the *Cat* mRNA expression and Cu/Zn SOD activity. Melatonin, in turn, markedly augmented the burn-induced Cu/Zn SOD ($P < 0.000\ 1$) activity, reversed the *Gpx4* mRNA ($P < 0.000\ 1$) and *Gsr* mRNA ($P < 0.000\ 1$) expression, and inhibited the *Cat* mRNA level. In conclusion, the present study suggests that a burn injury adaptively increases the Cu/Zn SOD activity and enhances the *Gpx4* and *Gsr* gene expression in the gastric mucosa. Melatonin effectively modulates the expression of the cellular antioxidant enzymes, and improves the antioxidant defence by augmenting the Cu/Zn SOD activity.

Keywords: gastric mucosa; oxidative stress; thermal injury

Severe burn trauma results in multiple organ dysfunction and is usually associated with increased morbidity and mortality rates. The most common failures, occurring after the skin injury, are haematological failure, respiratory failure, and cardiovascular failure (Feng et al. 2018). Complications also involve the gastrointestinal (GI) tract, and are commonly observed in burn injuries, in more than 20% of the total body surface area (TBSA). Problems manifest in the both upper and lower GI tract (Fadaak 2000). Clinical studies have shown that a thermal insult causes gastro-intestinal erosions, which can manifest with minor haemorrhages. Within 24–72 h after the trauma erosions,

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however, they may progress to Curling's ulcers. The latter, in turn, can lead to significant bleeding (Fadaak 2000; Bakkannavar et al. 2013).

Pharmacological prophylaxis is known to reduce burn-induced stress ulcers. In this regard, numerous experimental studies have been conducted to elucidate the pathophysiological mechanisms, responsible for burn-induced gastric mucosal injuries in order to prevent severe complications and improve the prognosis of the patients with burn trauma.

Studies indicate that oxidative stress causes the systemic response, as well as of the remote organ dysfunction in burn injuries (Toklu et al. 2007; Sehirli et al. 2008; Cevik et al. 2012). Oxidative stress is a phenomenon associated with the overproduction of radical oxygen species (ROS) and radical nitrogen species (RNS). Free radicals attack and damage a variety of cell components, including DNA, proteins and lipids. Oxidative stress has been a well-established factor for gastric mucosal injuries in burn trauma (Kabasakal et al. 2005; Sehirli et al. 2008). Such a condition requires participation of the antioxidant defence in order to prevent macromolecules from damage and maintain cellular homeostasis. The cellular antioxidant response is complex and involves collaboration between different defence levels. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) represent the first line defence antioxidants. These enzymes detoxicate the superoxide radicals, hydrogen peroxide and hydroperoxide converting them into harmless molecules (Ighodaro and Akinloye 2018). It was reported that experimental treatment with agents that enhance the antioxidant defence and restrict the oxidative processes in gastric mucosa exert a protective effect in burn-induced gastric mucosal injuries (Kabasakal et al. 2005; Sehirli et al. 2008).

Melatonin, a hormone produced in the pineal gland, has powerful antioxidant properties (Tan et al. 2015) that have been confirmed in thermal trauma. A melatonin application reduces malondialdehyde levels (MDA), prevents both lipid peroxidation and protein oxidation, and increases the reduced glutathione (GSH), CAT and SOD levels in liver, lung and intestinal tissues (Sener et al. 2002).

Melatonin has previously been shown to ameliorate gastric mucosal injuries via both induction of the antioxidant enzyme HO-1 and inhibition of the burn-induced oxidative stress (Hristova et al. 2016). It has also been shown to improve the antioxidant defence parameters in gastric tissues (Bekyarova et al. 2009).

However, the mechanisms of the possible gastroprotective effect of melatonin on gastric mucosal injuries induced by experimental burns has not been fully explained. Therefore, in this study, we aimed to investigate the role of Cu/Zn SOD, GPx, GR and CAT along with the gastroprotective properties of melatonin in a rat burn model.

MATERIAL AND METHODS

Experimental design

The experimental procedures were approved by the Home Office for Care and Use of Laboratory Animals and were performed with careful consideration of the ethics of animal experimentation according to the International Guiding Principles for Animal Research approved in Bulgaria (No. 90000088/2008).

A gastric mucosal injury was induced in a burn trauma model. Age-matched male Wistar rats (n = 18, weighing between 220–250 g), were maintained in individual wire-bottomed cages with paper pellet bedding under a controlled temperature (20 °C) and 12 : 12 light-dark cycle conditions with *ad libitum* access to rat chow and tap water.

For the experimental procedure, the animals were randomly assigned into one of three groups (n = 6 in each group) as follows: the control (C), i.e., the non-burned, non-treated group; the vehicle-treated burned group (B); and the melatonin-treated burned group (B + M). After light ether inhalation, general anaesthesia was performed using thiopental [30 mg/kg intraperitoneally (i.p.)]. In order to accomplish a third-degree burn over 30% of the total body surface area (TBSA), the dorsum of the rats was shaved, and exposed to 90 °C water bath for 10 seconds. For those rats that were subjected to the burn injury, 4 ml of physiological saline were applied(i.p. for immediate resuscitation following the burn injury. No animals died within the first 24 h of the post-burn period.

Either melatonin (*N*-acetyl-5-methoxytriptamine; Merck, Darmstadt, Germany) at a dose of 10 mg/kg body weight (b.w.) dissolved in a vehicle (2% ethyl alcohol diluted in physiological saline to consti-

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tute 5 ml/kg i.p.) was administered. Melatonin and the vehicle were applied immediately i.p. after the burns in the morning between 8:00 and 9:00 a.m. and 12 h after the thermal skin injury (Hristova et al. 2016). All the animals were given buprenorphine (0.3 mg/kg i.p. b.w.) twice daily for the pain to control the post-burn. They were re-anaesthetised with thiopental and euthanised 24 h after the burns so that the stomach could be sampled.

Paraffin processing of tissue

Tissue specimens of the gastric oxyntic mucosa were fixed in 10% buffered formalin (pH 7.2), dehydrated in an ascending series of ethyl alcohol (70–100%), and embedded in paraffin wax. Tissue sections with thicknesses of 5 µm were stained with haematoxylin and eosin (H&E) and examined using a light microscope (Olympus BH-2; Olympus, Tokyo, Japan). The histopathological changes were evaluated at a magnification of 200 × (a high-power field).

Immunohistochemistry

The immunohistochemistry protocol sections, deparaffinised and dehydrated, were treated with 1% hydrogen peroxide for the peroxidase activity inhibition for 5 minutes. Then, they were rinsed in 0.1 M phosphate buffered saline (PBS) (pH 7.4) and treated with normal goat serum for 20 minutes. Subsequently, the sections were incubated with the primary antibody for 24 h at room temperature. The antibodies Cu/Zn SOD (DAKO, Santa Clara, CA, USA) at a dilution of 1 : 50 were used. Finally, the peroxidase activity was estimated by the diaminobenzidine-tetrachloride H_2O_2 method. Negative controls were incubated with non-immune sera instead of the primary antibody.

A morphometric method was used to quantitatively assess the contents of the Cu/Zn SOD. The content was determined as strong with a score of 3, moderate with a score of 2, weak with a score of 1, or as lacking with a score of 0, on the basis of the occurrence of the immuno-deposits (Hristova et al. 2016). The Cu/Zn SOD concentration of the epithelial cells was defined as the content of each cell multiplied by their scoring factors, which was divided by the total number of cells. A morphometric investigation was performed on 50 cells from each sample. Two blinded observers counted the immuno-positive cells and the data were pooled.

RNA extraction

From each formalin-fixed paraffin-embedded (FFPE) tissue sample, 5 slices with 5 µm thickness were used for the RNA isolation. Deparaffinisation was performed by adding 1 ml of xylene to each sample, followed by mixing and centrifugation at 20 800 × *g*/2 min at room temperature. After removal of the xylene, the procedure was repeated. The residual xylene was washed by adding 1 ml of 100% ethanol. The ethanol was removed after brief mixing and centrifuged at 20 800 × *g*/2 min at room temperature. The procedure was repeated. The deparaffinised tissue was left to air dry and treated with 100 µl of a QuickExtract FFPE RNA extraction lysis buffer (Epicenter, Illumina, USA) according to the manufacturer's requirements, followed by additional treatment with 1 ml of a cold Accuzol solution (Bioneer, Daejeon, Republic of Korea) and incubation at room temperature for 8 minutes. Then 200 µl of chloroform was added to each sample and incubation at room temperature for 2 min followed by centrifugation at 12 000 × *g* at 4 °C/15 min was performed.

The aqueous phase was transferred to a sterile 1.5 ml microcentrifuge tube and mixed with an equal volume of absolute ethanol and transferred to RNA Clean and Concentrator 5 spin columns (Zymo Research, Irvine, CA, USA) for RNA purification according to manufacturer's protocol. Elution of the RNA was performed with 15 µl of nuclease free water and stored at –80 °C. The total RNA concentration was measured using a Take 3 microplate on a Synergy™ 2 Multi-Mode Microplate Reader (Biotek,Winooski, VT, USA).

cDNA synthesis

For the cDNA synthesis, 20 ng of a total RNA RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) was used, applying the protocol for the oligo dT priming strategy according to the manufacturer's requirement. The polymerase chain reaction (PCR) was performed on the GeneAmp® PCR System 9700

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Table 1. Sequences of the primers used in qPCR analyses

Gene	Forward 5'-3'	Reverse 5'-3'
Actin beta (*Actb*)	ACCGTGAAAAGATGACCCAG	CAGAGGCATACAGGGACAAC
Glutathione peroxidase 4 (*Gpx4*)	TGGTTTGCCTGGATAAGTACAG	GTTTACGTCGGTTTTGCCTC
Glutathione-disulfide reductase (*Gsr*)	CATCAAGGAGAAGCGGGATG	CGATGTGGGACTTGGTTAAATTG
Catalase (*Cat*)	ACAACTCCCAGAAGCCTAAGAATG	GCTTTTCCCTTGGCAGCTATG

(Applied Biosystems, Waltham, MA, USA). The synthesised cDNA samples were diluted with 30 µl nuclease free water and stored at –20 °C.

Real-time PCR

The real-time PCR (qPCR) analyses were performed using KAPA SYBR® Fast qPCR Kit (Kapa Biosystems, Cape Town, South Africa) according to the manufacturer's requirements with the addition of dissociation curve analyses. The qPCR was performed on the ABI 7500 Real Time PCR System (Applied Biosystems, Waltham, MA, USA). Actin beta was used as the endogenous control. The primer sequences used for the analyses are presented in Table 1. All the analyses were performed in triplicate. The gene expression levels were presented as relative units (RU) mRNA, normalised against actin beta as the endogenous control gene. Calculation of the mRNA expression levels were undertaken using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

The statistical analysis was conducted using GraphPad Prism v6.0 software (La Jolla, CA, USA). The data are presented as mean ± SEM. The statistical significance of the difference was evaluated with the Mann-Whitney *U* test and Student's *t*-test. A level of $P < 0.05$ was considered significant.

RESULTS

The melatonin effect on the Cu/Zn SOD protein expression in the gastric mucosa

Cu/Zn SOD-positive cells were localised in all parts of the gastric mucosa, but mainly in its upper half in the control group. The intensity of the staining for Cu/Zn SOD was strongly expressed in the cells of the upper half of the gastric mucosa and weakly expressed in its basal parts (Figure 1A). The content was 1.02 ± 0.078. In the burned group, ex-

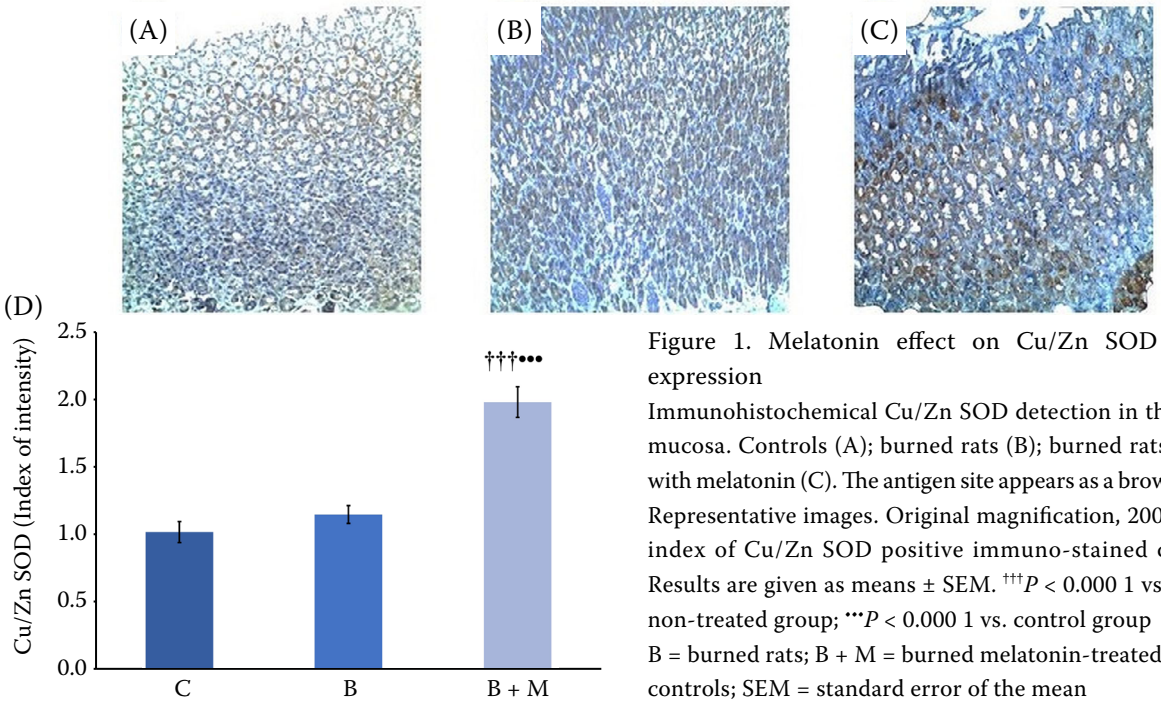

Figure 1. Melatonin effect on Cu/Zn SOD protein expression

Immunohistochemical Cu/Zn SOD detection in the gastric mucosa. Controls (A); burned rats (B); burned rats, treated with melatonin (C). The antigen site appears as a brown colour. Representative images. Original magnification, 200 ×. Score index of Cu/Zn SOD positive immuno-stained cells (D). Results are given as means ± SEM. †††$P < 0.000\,1$ vs. burned, non-treated group; •••$P < 0.000\,1$ vs. control group

B = burned rats; B + M = burned melatonin-treated rats; C = controls; SEM = standard error of the mean

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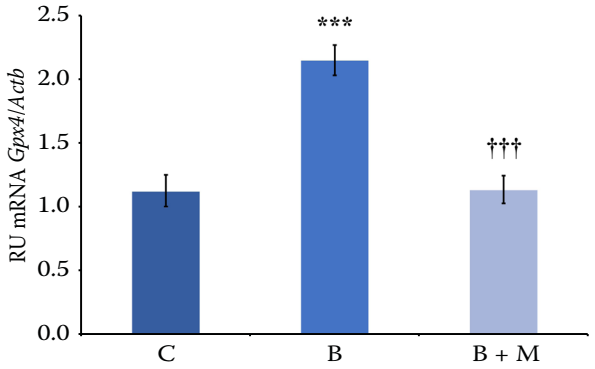

Figure 2. Melatonin effect on the *Gpx4* mRNA expression
Expression of *Gpx4* mRNA in the gastric mucosa was measured using qPCR. Results are given as RU mRNA means ± SEM. $^{***}P < 0.000\,1$ vs. control group; $^{†††}P < 0.000\,1$ vs. burned, non-treated group
Actb = actin beta; B = burned rats; B + M = burned melatonin-treated rats; C = controls; *Gpx4* = glutathione peroxidase 4; qPCR = real-time PCR; RU = relative units; SEM = standard error of the mean

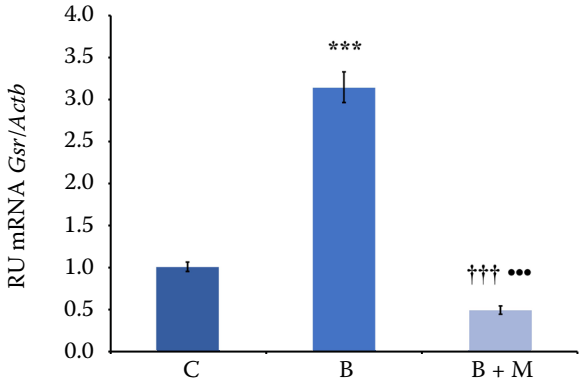

Figure 3. Melatonin effect on the *Gsr* mRNA expression
Expression of *Gsr* mRNA in the gastric mucosa was measured using qPCR. Results are given as RU mRNA means ± SEM. $^{***}P < 0.000\,1$ vs. control group; $^{†††}P < 0.000\,1$ vs. burned, non-treated group; $^{•••}P < 0.000\,1$ vs. control group
Actb = actin beta; B = burned rats; B + M = burned melatonin-treated rats; C = controls; *Gsr* = glutathione-disulfide reductase; qPCR = real-time PCR; RU = relative units; ; SEM = standard error of the mean

pression of the Cu/Zn SOD was found in almost all the epithelial cells, while diffuse in the gastric mucosa. The reaction intensity was low (Figure 1B). The mean Cu/Zn SOD protein content was increased by 13% (1.15 ± 0.066) compared to the control group. In the burned melatonin-treated group, the Cu/Zn SOD expression was present in almost all the cells, and varies from being strong in the upper portion to moderate at the basal region of the gastric mucosa (Figure 1C). Its reaction intensity was 1.98 ± 0.11. The Cu/Zn SOD expression was higher compared to both groups [72% vs. burned group ($P < 0.000\,1$) and 94% vs. control group ($P < 0.000\,1$)].

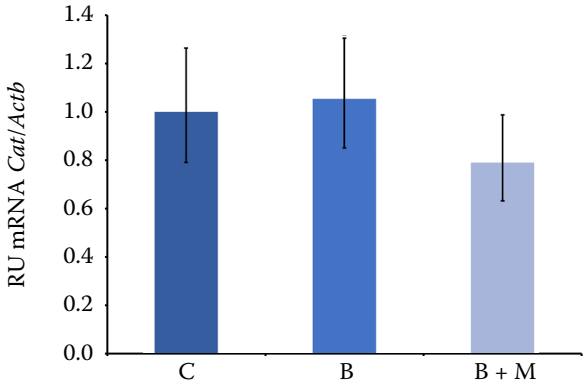

Figure 4. Melatonin effect on the *Cat* mRNA expression
Expression of *Cat* mRNA in the gastric mucosa was measured using qPCR. Results are given as RU mRNA means ± SEM
Actb = actin beta; B = burned rats; B + M = burned melatonin-treated rats; C = controls; Cat = catalase; qPCR = real-time PCR; RU = relative units; SEM = standard error of the mean

The melatonin effect on the gene expression in the gastric mucosa

In the burned group, the glutathione peroxidase 4 (*Gpx4*) gene expression was significantly increased by 92% ($P < 0.000\,1$) compared to the control group. The melatonin treatment notably decreased the elevated gene expression by 47.4% ($P < 0.000\,1$) compared to the burned group reaching the control values (Figure 2).

Similarly, the glutathione-disulfide reductase (*Gsr*) gene expression in the burned group was significantly increased by 212% ($P < 0.000\,1$) compared to the control group. Following the melatonin treatment, however, the *Gsr* gene expression was remarkably diminished by 84.43% ($P < 0.000\,1$) and 51.39% ($P < 0.000\,1$), compared to the burned group and the control group, respectively (Figure 3).

In contrast to the control group, the burned group demonstrated a negligible catalase (*Cat*) gene expression level. The melatonin treatment reduced the *Cat* gene expression by 24.94% compared to the burned group, and by 20.89% than that of the control group (Figure 4).

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DISCUSSION

Severe burn injuries have been associated with both local skin damage and systemic complications. It is a stressful condition, which can represent challenges to various homeostasis mechanisms (Sehirli et al. 2008). ROS/RNS overproduction is considered one of the key moments in distant-organ injuries (Szczesny et al. 2015; AbuBakr et al. 2018). Ischaemia and reperfusion injury (I/R) in the splanchnic vascular bed along with the supervene reduction of the gastric blood flow are triggering factors for the excessive production of ROS and RNS (Kamada et al. 1982). In addition, increased xanthine oxidase activity, primed leukocyte infiltration, and decreased energy production and mitochondrial dysfunction contribute to the oxidant gastric injury in burns (Zhu et al. 2000; Jacob et al. 2017). There is a balance between the formation of oxygen free radicals and antioxidant defence systems. When excessive ROS/RNS production disrupts this balance, this results in oxidative stress and damage to the biomolecules (Ighodaro and Akinloye 2018). Research has shown that burn injuries elevate, in the gastric mucosa, both the MDA levels, a sure marker for lipid peroxidation, and the inducible nitric oxide synthase (iNOS) levels, which, in turn, are a source of peroxynitrite, a powerful and toxic oxidant (Hristova et al. 2016).

In the present study, we found a significant increase in the expression of *Gpx4* mRNA, *Gsr* mRNA, and negligible elevation in the Cu/Zn SOD protein and *Cat* mRNA expression in the gastric tissue of rats exposed to thermal trauma.

Regarding the tissue enzyme activity in thermal trauma, the results are scarce and contradictory. Our findings are in line with Saitoh et al. (2001) who reported that a burn injury increases the Mn SOD or Cu/Zn SOD isoforms, and varies according to the type of tissue. Other authors reported low or even absent SOD activity in the organs (Cevik et al. 2012; AbuBakr et al. 2018).

It is known that exogenous SOD has a protective effect in oxidative gastric mucosal injuries (Konturek et al. 2000). The therapeutic potential of the enzyme was also documented in burns. The prophylactic administration of SOD derivative inhibits the lipid peroxidation in the blood plasma and tissues, and increases the survival rate in the injured group (Saitoh et al. 1994).

As a first line defence antioxidant, cytosolic Cu/Zn SOD has the role to directly neutralise superoxide radicals, which initiate the process of free radical generation in the cells. We hypothesise that the increased Cu/Zn SOD activity in the gastric mucosa serves as a compensatory response to burn injuries. Most probably, this occurs through the inhibition of superoxide radicals and cytotoxic peroxynitrite overproduction, resulting from the superoxide anion radical ($O_2^{\bullet -}$) and nitric oxide (NO)/iNOS interaction (Hristova et al. 2016).

Reduced glutathione (GSH) is the key non-enzymatic antioxidant in the body. Cellular homeostasis of this antioxidant is not only regulated by its *de novo* synthesis, but also has been associated with GSH – related antioxidant enzymes, such as GPx and GR. GPx detoxifies peroxides with GSH acting as an electron donor in the reduction reaction, producing glutathione disulfide (GSSG) as an end product. The reduction of GSSG is catalysed by GR (Lushchak 2012).

Catalase is one of the most important antioxidant enzymes. It catalyses the degradation or reduction of hydrogen peroxide to water and molecular oxygen, consequently completing the detoxification process, imitated by SOD (Ighodaro and Akinloye 2018).

Studies have shown that burn injuries decrease the CAT activities, and elevate that of the GPx in the tissues (Cevik et al. 2012; AbuBakr et al. 2018). More recent studies have revealed that the GSH concentration, and the synthesis rates are significantly reduced in the whole blood and tissues after burn injuries (Fei et al. 2013). Low GSH levels were also reported in the gastric tissue following skin trauma (Kabasakal et al. 2005; Sehirli et al. 2008).

In our study, the increased glutathione-related enzymes and CAT expression may have two possible explanations. Firstly, the elevated SOD expression boosts the hydrogen peroxide production, an enzyme substrate for both GPx and CAT. Secondly, it implies a remarkable elevation in the *Gpx4* mRNA expression, providing the substrate for GR, hence increasing the *Gsr* mRNA expression. All the mentioned results correlate with previous research on diminished gastric GSH levels after burn injuries (Kabasakal et al. 2005; Sehirli et al. 2008). Generally, the markedly increased gene expression of the glutathione-related antioxidant enzymes and elevated SOD levels suggest that thermal trauma triggers the processes of cellular homeostasis in the gastric tissue.

Burn-induced oxidative stress is accompanied by histological changes in the gastric mucosa such

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as epithelial degeneration, vasocongestion and severe oedema in the lamina propria, and leukocyte infiltration. In addition, the use of antioxidant compounds attenuate the oxidative stress, and has a beneficial effect on the morphological changes in the gastric tissue (Kabasakal et al. 2005; Sehirli et al. 2008).

Melatonin has a pleiotropic biological effect, however, one of the main peripheral functions of melatonin is its antioxidant function. As a highly effective antioxidant, it is known to activate the expression of antioxidant enzymes and to indirectly capture and detoxify ROS/RNS. Melatonin clears up to 10 ROS compared to classic antioxidants. Melatonin products also take an active part in the detoxification process. These events reflect its cascade reaction with ROS and nitric oxide synthase (NOS) (Tan et al. 2015). The high antioxidant potential of melatonin is determined by its lipophilic structure and the ability to easily pass through the cell membranes (Costa et al. 1995). As an important endogenous protector and participant in the homeostasis of the body, it also prevents damage to the gastric mucosa induced by stress and I/R (Bandyopadhyay et al. 2000; Cabeza et al. 2001). Tissue protective action has been established experimentally, both locally and in distant organs in thermal injuries (Sener et al. 2002; Basak et al. 2003).

Gastro protection via ameliorating the oxidative stress by decreasing the MDA and increasing the total thiol levels have also been reported for melatonin (Bekyarova et al. 2009).

Our data demonstrate that melatonin significantly increases the Cu/Zn SOD expression in the gastric mucosa in the first 24 h following a burn injury on one hand, and maintains the expression of *Gpx4* mRNA, *Gsr* mRNA, closer to that of the controls, and the *Cat* mRNA expression lower compared to the controls, on the other hand. Through an increase in the SOD activity, the first line defence antioxidant, melatonin provides a more balanced antioxidant enzyme protection against burn-induced oxidative injury. Based on these results, we hypothesise that the defensive effect of melatonin in a burn-induced gastric mucosal injury is predominantly due to the increased SOD activity, successful superoxide radical detoxification, along with the limiting production of other ROS/RNS.

In addition, the observed effects on the GPx, Gsr and Cat are most likely due to the direct capture of free radicals, produced in the burn experimental model. It is well known that melatonin can directly neutralise the variety of ROS, including peroxides (Tan et al. 2015). Its presence could be a reason for the decreased requirements for the respective enzyme synthesis. Therefore, it is reasonable to suggest that such an ROS scavenging activity could underlie the observed reducing effect on the *GPx* and *Gsr* expression. Although not significant, the observed *Cat* expression reduction contributes to this hypothesis.

To summarise, the present study revealed a significant increase in the expression of Cu/Zn SOD, *Gpx4* mRNA, *Gsr* mRNA in the gastric mucosa, which probably accounts for a compensatory response to the overproduction of free radical species in severe burn injuries. Melatonin provides a more balanced antioxidant enzyme protection against burn-induced oxidative injuries of the gastric mucosa. Melatonin augments the Cu/Zn SOD expression, and by inhibiting the production of other free radicals, acts as a natural inducer of the gastric mucosal protection against a burn-induced oxidative injury.

This study complements previous studies, regarding the cellular antioxidant mechanisms, stimulated by melatonin, as a new gastro-protector in thermal trauma.

Conflict of interest

The authors declare no conflict of interest.

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