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Protective effects of melatonin against oxidative damage induced by Egyptian cobra (*Naja haje*) crude venom in rats

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ABSTRACT

Naja haje envenomation is one of the leading causes of death due to snakebite. Antiserum therapy sometimes fails to provide enough protection against venom toxicity. In this study, we investigated the protective effects of melatonin against *N. haje* venom in rats. The animals were injected with venom (0.25 mg/kg) and/or melatonin (10 mg/kg) and compared with vehicle-treated rats. There was oxidative/nitrosative damage and apoptosis in the liver, heart, and kidneys of venom-injected rats. Melatonin counteracted the increased lipoperoxidation and nitric oxide, prevented decreased glutathione peroxidase and reductase activity, reduced the glutathione disulfide/glutathione (GSSG/GSH) ratio, and maintained the GSH pool. Furthermore, melatonin administration was associated with a reduction of apoptosis, which was increased in venom-injected rats. Overall, these results suggest that melatonin mitigates oxidative/nitrosative stress in venom-induced cardio-hepato-renal injury in rats. Our results suggest that melatonin treatment may ameliorate some of the effects of *N. haje* envenomation.

1. Introduction

Snakebite is a critical public health problem faced by many tropical countries (Chippaux, 1998). It has been reported that over 1 million humans are bitten annually by various types of snakes, resulting in more than 95,000 deaths (Mohapatra et al., 2011).

Local necrosis and hemorrhagic lesions can occur after snake envenomation (Gutierrez et al., 2006). Hemorrhage damages the vascular endothelium, leading to bleeding from vital organs. The lethal effects of snake venom are largely attributed to phospholipase A2s (PLA2s), which are commonly found in snake venoms. These enzymes hydrolyze phospholipids, resulting in the formation of lipid peroxides and subsequent lipid peroxidation (LPO). The PLA2s in venoms have been implicated in multiple pathologies, including hepatotoxicity (Mukherjee and Maity, 1997), cardiotoxicity, (Cher et al., 2005) and nephrotoxicity (de Castro et al., 2004a). Furthermore, a complex array of venom cytotoxins penetrate the cells (Yap et al., 2014) and interact with intracellular targets, such as the mitochondria (Wang and Wu, 2005). Mitochondrial impairment can produce reactive oxygen/nitrogen species (ROS/RNS) that in turn reduce mitochondrial bioenergetics, leading to cell damage and death (Escames et al., 2012). The persistence of oxidative stress damages vital organs, such as the liver and kidneys, resulting in multiple organ failure.

Antiserum, which contains antibodies against some of the components of snake venom, is the only agent available to treat envenomation; it appears to ameliorate some of the toxic effects of venom but fails to provide protection against venom-induced necrosis, hemorrhage, and renal failure (Venkatesan et al., 2014). Moreover, antiserum is often associated with hypersensitivity reactions that may be lethal (Devi et al., 2002), and it is expensive and thus out of reach of many patients in rural areas. Moreover, a lack of information about the biting species in most cases and the correct antiserum dosage and stability restricts its efficacy (Shashidharamurthy et al., 2010). Therefore, there are clinical and economic reasons to identify novel and effective therapies for snakebite. Melatonin (*N*-acetyl-5-methoxytryptamine) is an indolamine produced by the pineal gland (Lerner et al., 1958)

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and by many other peripheral tissues and organs (Stefulj et al., 2001; Venegas et al., 2012; Acuña-Castroviejo et al., 2014). It is a potent free radical scavenger that possesses antioxidant and anti-inflammatory properties (Poeggeler et al., 1994; Tan et al., 1998; Crespo et al., 1999; Tan et al., 2001; Escames et al., 2006; Tan et al., 2007; Reiter et al., 2009; Galano et al., 2011; Galano, 2013). When scavenging free radicals, melatonin is transformed into a series of metabolites that are also free radical scavengers (Hardeland et al., 2009).

In addition to its direct scavenger activity, melatonin has a genomic effect, inducing the expression of antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GRd), and superoxide dismutase (SOD) (Antolin et al., 1996; Rodriguez et al., 2004). Melatonin also exhibits immunomodulatory properties, such as inhibiting the expression of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) and reducing inflammation (Crespo et al., 1999; Deng et al., 2006).

Melatonin has been shown to exert marked anti-inflammatory effects against crude venom of the jellyfish *Pelagia noctiluca* by inhibiting apoptosis and attenuating the expression of iNOS (Marino et al., 2009). It also mitigates the oxidative stress and vital organ damage induced by venom of the viper *Echis carinatus* in mice (Katkar et al., 2014). Because of the previously reported protective effects of melatonin against venom from a variety of sources, we investigated whether melatonin would be effective against *N. haje* venom-induced toxicity in the liver, kidneys, and heart of rats.

2. Materials and methods

2.1. Venom source

Pooled venom samples from *N. haje* were milked from 10 specimens collected from the West Delta in Egypt. The snake venom was diluted in deionized water and centrifuged at 10,000×*g* for 15 min; the pellet was discarded. The supernatant was dried under vacuum and stored at −20 °C. Before use, the venom was reconstituted in saline and centrifuged at 3000×*g* for 10 min at 4 °C; the supernatant was used in the present study. The concentration of venom is expressed in terms of dry weight.

The LD_{50} (mg/kg body weight of mice) was determined by the method of Meier and Theakston (1986) and was found to be 0.25 mg.

2.2. Animals

All of the experiments were conducted in accordance with the University of Granada's Ethical Committee, the Spanish (R.D. 53/2013) and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS # 123), and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Sciences, Bethesda, MD, USA). Three-month-old male Wistar rats (220–250 g) obtained from Harlan Laboratories (Barcelona, Spain) were maintained in the University of Granada and housed in wire-bottomed cages under 12-h light–dark cycles (lights on at 08:00 am) at 25 ± 1 °C. The rats were provided with water and a balanced diet ad libitum.

2.3. Experimental protocol

To study the effect of the venom LD_{50} on the heart, liver, and kidneys, rats were randomly assigned into the following three groups ($n = 7$ per group): control (vehicle-treated rats), venom (intraperitoneal injection of venom), and venom + melatonin (intraperitoneal injection of melatonin and venom). Melatonin was administered 20 min before the venom to ensure that it reached all of the tissues. The rats were killed 5 h after the venom or vehicle was administered. They were first anesthetized with halothane and then killed by cervical dislocation. The heart, liver, and kidneys were rapidly removed, washed in cold saline, and processed for histological assessment or stored at −80 °C until they were used.

2.4. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity was determined by using a spectrophotometer to follow the decrease in NADH at 340 nm (Shvedova et al., 2013). LDH activity is expressed as U/mg protein.

2.5. Nitrite determination

Tissues were weighed and homogenized in ice-cold distilled water. The homogenate was centrifuged at 800×*g* for 10 min at 4 °C. Aliquots of the supernatant were either stored at −80 °C for total protein determination (Lowry et al., 1951) or used to calculate nitrite (NOx) levels following the Griess reaction (Green et al., 1981). The amount of nitrite in the supernatant was measured following the Griess reaction by incubating 100 μl of sample with 100 μl of Griess reagent (0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride; 1% sulfanilamide in 5% phosphoric acid; 1:1) at room temperature for 20 min. The absorbance at 550 nm was measured with a Bio-Tek EL 800 microplate reader (IZASA, Seville, Spain). Nitrite concentration was calculated by comparison with the absorbance of a standard solution of known sodium nitrite concentration, and the results are expressed as nmol nitrite/mg protein.

2.6. Lipid peroxidation measurement

Tissues were weighed and homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at 800×*g* for 10 min at 4 °C. Aliquots of the supernatant were used for total protein determination (Lowry et al., 1951) or used for LPO measurement with a commercial kit (Bioxytech LPO-568 assay kit; OxisResearch, Portland, OR, USA) able to determine both malondialdhehyde (MDA) and 4-hydroxy-2(*E*)-nonenal (4-HNE) (Esterbauer and Cheeseman, 1990) based on the condensation reaction of the chromogene 1-methyl-2-phenylindole with either MDA or 4-HNE. The stable chromophores were determined at 586 nm. LPO levels are expressed as nmol/mg protein.

2.7. Measurement of glutathione and glutathione disulfide

Glutathione (GSH) and glutathione disulfide (GSSG) were measured by an established fluorometric method (Hissin and Hilf, 1976). Tissues were weighed and homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at 800×*g* for 10 min at 4 °C. Aliquots of the supernatant were deproteinized with ice-cold 10% trichloroacetic acid and centrifuged at 20,000×*g* for 15 min. For GSH measurement, supernatant aliquots were incubated with an *ortho*-phthalaldehyde/ethanol solution (1 mg/mL) and phosphate buffer (100 mM sodium phosphate and 5 mM EDTA-Na_2, pH 8.0) at room temperature for 15 min. The fluorescence of the samples was then measured at 340 nm excitation and 420 nm emission wavelengths in a plate-reader spectrofluorometer (Bio-Tek Instruments, Inc., Winooski, VT, USA).

For GSSG measurement, aliquots of supernatants were preincubated with *N*-ethylmaleimide solution (5 mg/ml) at room temperature for 40 min and then alkalinized with 0.1 N NaOH. Aliquots of these mixtures were then incubated with *ortho*-phthalaldehyde solution (1 mg/ml) and 0.1 N NaOH at room

temperature for 15 min. The fluorescence was then measured. GSH and GSSG concentrations are expressed as nmol/mg protein.

2.8. Measurement of glutathione peroxidase and glutathione reductase activities

Tissues were thawed, suspended in buffer A (potassium-phosphate 50 mM and EDTA-K2 1 mM, pH 7.4), and homogenized. To measure GPx activity, samples were added to a working solution containing buffer A plus 4 mM sodium azide, 4 mM GSH, 0.2 mM NADPH, and 0.5 U/ml GRd. After incubation at 37 °C for 4 min, the reaction was started with the addition of cumene hydroperoxide (0.3%) and GPx activity was determined by following the oxidation of NADPH at 340 nm for 3 min in a UV spectrophotometer (Shimadzu Deutschland GmBH, Duisburg, Germany) (Jaskot et al., 1983). GRd activity was measured in samples added to a working solution containing buffer A plus 2 mM GSSG. After incubation at 37 °C for 4 min, the reaction was started by adding 9.5 mM NADPH solution and GRd activity was measured following the oxidation of NADPH at 340 nm for 3 min. In both cases, nonenzymatic NADPH oxidation was subtracted from the overall rates. GPx and GRd activities are expressed as nmol/min/mg protein.

2.9. Western blot analysis

Tissues were thawed and homogenized with a Polytron homogenizer (Kinematic, Switzerland) at 4 °C in HEPES buffer (20 mM, pH 7.4) containing 0.02% (w/v) bacitracin, 0.4 mM PMSF, 1 mM benzamidine, 1.5 μM pepstatin, and 0.1 mM aprotinin. The homogenates were then centrifuged at 4000×*g* for 10 min at 4 °C, and supernatants were collected to assay the protein content (Lowry et al., 1951) and expression. For protein expression assays, samples (75 μg) were eluted from the supernatant directly into sodium dodecyl sulfate (SDS) sample buffer, applied to 7.5% SDS–PAGE gels, and transferred onto nitrocellulose membranes (Hoeffer, San Francisco, CA, USA). The membranes were then incubated in blocking

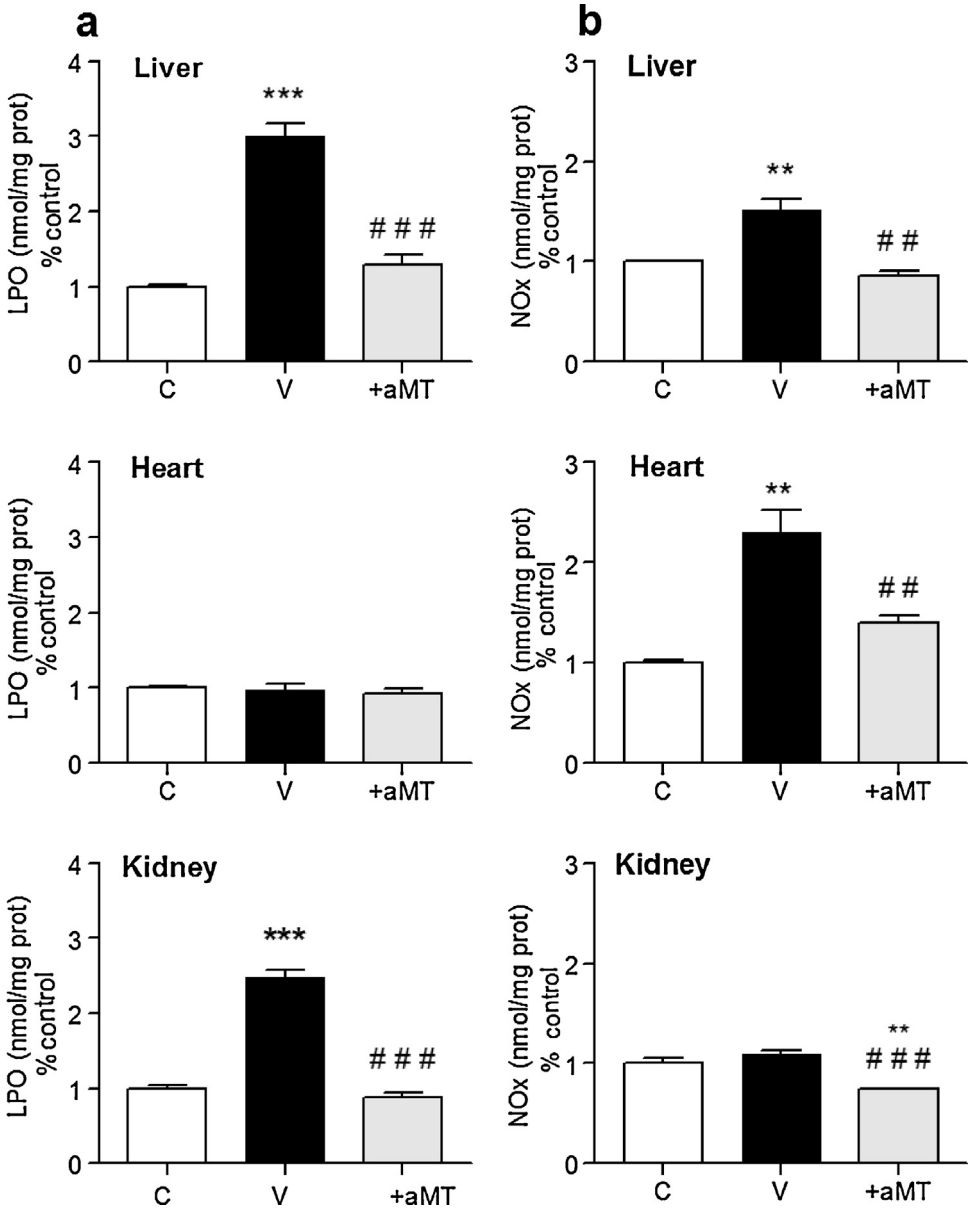

Fig. 1. Lipoperoxide (LPO) (a) and NOx (b) levels in homogenates of liver, heart, and kidney tissue from control rats (C), rats treated with venom (V), and rats treated with venom + melatonin (+aMT). Data are expressed as mean ± SEM ($n = 6$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. venom.

buffer (PBS, 0.1% Tween 20 and 5% dry milk) and shaken at room temperature for 2 h. Once the blocking process was complete, the membranes were incubated with anti-Bax rabbit antibody (1:200, SC526; Santa Cruz Biotechnology) or anti-Bcl2 rabbit antibody (1:200, SC492; Santa Cruz Biotechnology) at 4 °C overnight. The next day, membranes were washed and incubated with 1:5000 horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (H+L) (31460; ThermoScientific) at room temperature for 60 min. The proteins were visualized with a chemiluminescence kit (Western Lightning Plus-ECL; Perkin-Elmer, Billerica, MA, USA) according to the manufacturer's protocol and analyzed with Kodak equipment (Image Station 2000R; Eastman Kodak Company, Rochester, NY, USA).

2.10. Histological studies

Samples were fixed in 10% formaldehyde 3.7–4% buffered to pH 7 and stabilized with methanol DC (Panreac, Barcelona, Spain) for 48 h and then embedded in paraffin. Multiple sections (4 μm thickness) were deparaffinized with xylene and stained with hematoxylin and eosin (H&E).

2.11. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). Duncan's test was used as a post-hoc test to determine significant differences between groups. The software program SPSS version 17.0 was used for the analyses.

3. Results

3.1. Oxidative stress

Venom administration was associated with a significant increase in LPO in the liver and kidneys, but not in the heart, of

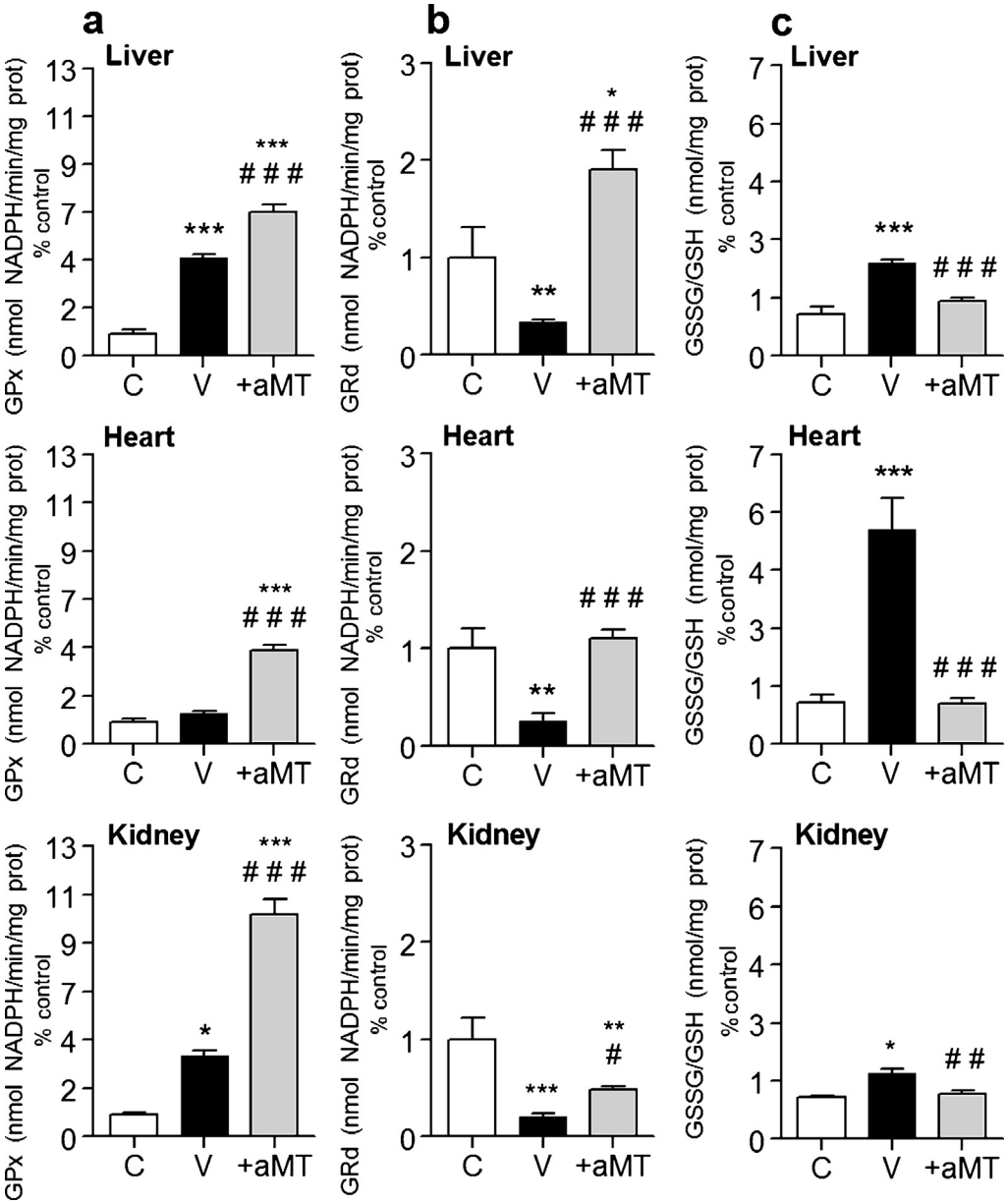

Fig. 2. GPx (a) and GRd (b) activities, and GSSG/GSH in homogenates of liver, heart, and kidney tissue from control rats (C), rats treated with venom (V), and rats treated with venom + melatonin (+aMT). Data are expressed as mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. venom.

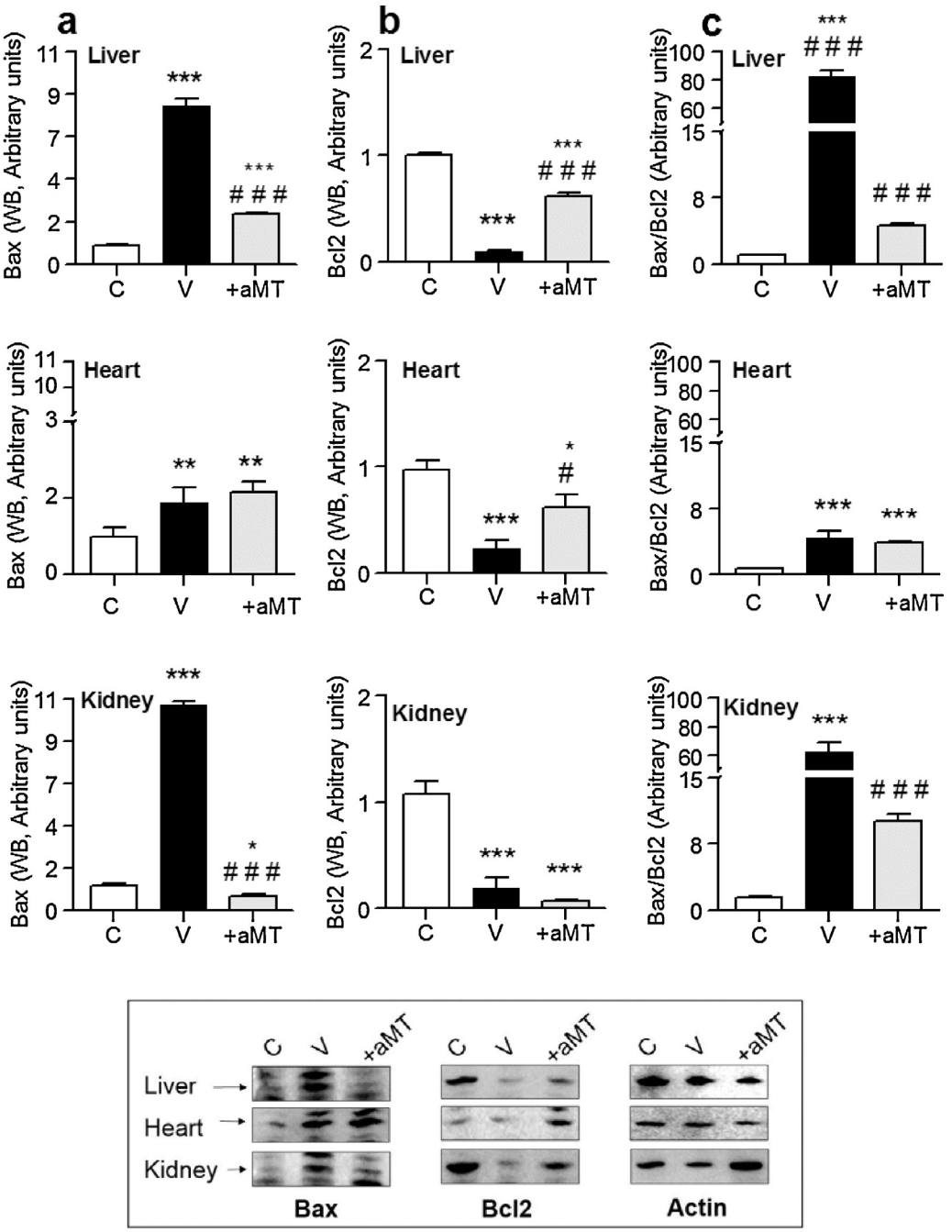

Fig. 3. Western blot (WB) analysis and densitometric quantification of Bax (a), Bcl2 (b), and Bax/Bcl2 ratio (c) in homogenates of liver, heart, and kidney tissue from control rats (C), rats treated with venom (V), and rats treated with venom + melatonin (+aMT). Data are expressed as mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. $^{\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. venom.

the rats (Fig. 1a). In rats that were administered melatonin, LPO levels were similar to those of the vehicle-treated rats.

After venom administration, only the liver and heart, but not the kidneys, showed a significant increase in NOx (Fig. 1b), a marker for NO• levels. Melatonin administration counteracted these effects and even reduced NOx levels to below the basal values in kidney tissue.

Venom injection increased the activity of GPx in the liver and kidneys, but not in the heart (Fig. 2a), reflecting the changes in LPO in these organs (Fig. 1a). Melatonin increased the activity of GPx to above that of the controls (Fig. 2a). In contrast, in rats administered venom alone, GRd activity in the liver, heart, and kidneys was reduced. This effect was completely prevented by melatonin administration (Fig. 2b). These findings reflect the oxidizing response of these tissues to venom exposure, which was further supported by the increase in the GSSG/GSH ratio (Fig. 2c). The data also support the antioxidant activity of melatonin, which increased GSH and normalized the GSSG/GSH ratio (Fig. 2c).

3.2. Apoptosis and cytotoxicity

The effects of *N. haje* snake venom and melatonin administration on Bax and Bcl2 levels are shown in Fig. 3. Venom upregulated Bax and downregulated Bcl2 protein levels, increasing the Bax/Bcl2 ratio in the liver, heart, and kidneys (Fig. 3a–c, respectively). These effects were counteracted by melatonin administration, which

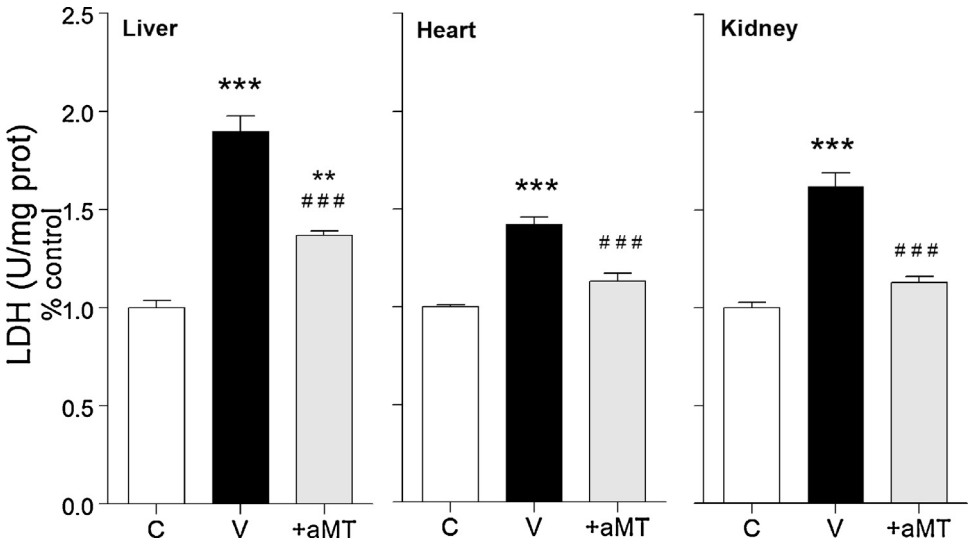

Fig. 4. LDH activity in homogenates of liver, heart, and kidney tissue from control rats (C), rats treated with venom (V), and rats treated with venom + melatonin (+aMT). Data are expressed as mean ± SEM ($n = 6$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. $^{###}P < 0.001$ vs. venom.

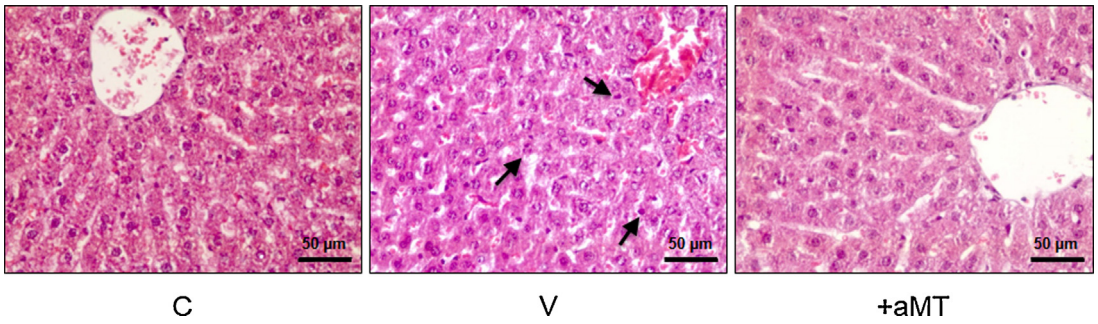

Fig. 5. H&E staining of liver from control rats (C), rats treated with venom (V), and rats treated with venom + melatonin (+aMT). Necrotic cells are indicated with black arrows.

decreased Bax levels and increased Bcl2 levels. Moreover, LDH activity, a biomarker of tissue damage, increased with venom administration, and melatonin counteracted this effect (Fig. 4).

3.3. Histopathology

H&E staining revealed significant histological changes in the liver (Fig. 5), but not in the other tissues (data not shown), after venom injection. Degenerated hepatocytes with reduced staining were evident adjacent to the centrolobulillar vein. There were also hemorrhagic regions and a loss of normal morphology of the sinusoids due to infiltration by pleomorphic cells. There was also an increase in necrotic cells. In the melatonin-treated group, there were significantly fewer histopathological lesions compared to the venom group. We observed only a slight reduction in staining around the centrolobulillar vein.

4. Discussion

According to the results of our study, melatonin may have significant protective effects against Egyptian cobra snake venom.

Oxidative stress and cellular toxicity appear to be involved in the multiple organ damage that follows snake envenomation. We selected melatonin as a potential therapeutic agent to neutralize *N. haje* venom because of its cell-protecting role in the body (Martin et al., 2000a; Acuña-Castroviejo et al., 2011). In the present study, snake venom increased LPO in the liver and kidneys and NO• levels in the liver and heart. These results may explain the multiple organ dysfunction observed following snake poisoning (Ali et al., 1981; de Castro et al., 2004b; Al et al., 2006). The increased LPO in various organs following venom injection may depend upon the increased availability of fatty acids, which are mobilized from adipose tissue (El-Asmar et al., 1979). Treatment with melatonin significantly ameliorated the increase in LPO and NO• levels induced by venom. The protective effects of melatonin have also been demonstrated under various conditions of oxidative stress (Garcia et al., 2014) including1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced oxidative damage in nigrostriatal neurons (Sewerynek et al., 1995; Khaldy et al., 2003) and gentamicin-induced nephrotoxicity (Sewerynek et al., 1995; Khaldy et al., 2003).

To counteract excessive ROS, cells have an antioxidant system that includes the GSH cycle. We found that venom increased the activity of GPx while reduced the activity of GRd, impeding recovery of the GSH pool, which is required for normal GSH cycle functions (Martin et al., 2000b). These findings reflect an oxidizing response to venom, further supported by the increase in the GSSG/GSH ratio (Griffith, 1999), which was more evident in the liver and heart than in the kidneys. Melatonin increased GRd and GPx activity, which resulted in normalization of the GSSG/GSH ratio in all organs tested. Thus, melatonin appears to protect lipids against venom-induced oxidation via ROS/RNS scavenging and the antioxidant activity of indolamine (Acuña-Castroviejo et al., 2011).

Generation of ROS and NO• causes defects in mitochondrial function associated with the induction of apoptosis (Garcia-Corzo et al., 2013). In the current report, venom upregulated the pro-apoptotic protein Bax and downregulated the anti-apoptotic protein Bcl2 in all of the studied tissues. These conditions favor the

opening of the mitochondrial permeability transition pore, releasing cytochrome c to the cytosol and activating caspase-3, implying that a caspase-dependent cell death pathway was activated. The antiapoptotic effect of melatonin observed in this study is probably linked to its function in reducing oxidative damage (Chen and Chuang, 1999). Moreover, some snake venoms contain toxins that induce cell death in vivo and selectively damage certain types of cells (Mukherjee and Maity, 2002). In the present study, LDH activity was significantly higher in tissue from envenomated rats compared to vehicle-treated rats, while melatonin administration counteracted these effects. However, histological observation of the tissues of envenomated rats only revealed significant histological changes in the liver. We observed necrotic cells and congestion of blood vessels, which may cause liver dysfunction (Venkatesan et al., 2014). In small foci of necrosis, typically cells are swollen and there is loss of membrane integrity; usually not rounded and less intensely stained than apoptotic bodies. However, in apoptosis, affected hepatocytes have condensed hypereosinophilic cytoplasm and a somewhat outline. Also, apoptotic bodies are usually rounded with condensed cytoplasm and typically surrounded by a clear halo. In the picture, the arrowed cells not present hypereosinophilic cytoplasm, the cell membrane is lost and apoptotic bodies are not present.

Venom showed variable cytotoxic effects against the different tissues that we studied. This variability may be due to the fact that snake venoms contain various enzymes and toxic proteins that represent the most sophisticated integrated weapons systems in the natural world (Fry, 2005). Moreover, there are marked differences in the effects of different cytotoxins on various mammalian cell types (Konshina et al., 2011). The complexity of in vivo systems often makes it difficult to interpret effects measured at the molecular level.

In conclusion, our results provide evidence of the beneficial effects of melatonin in combating snake venom-induced cellular damage. The lack of toxicity of melatonin even at high doses and with chronic administration (Rodriguez et al., 2007; Kolli et al., 2013) makes it of interest for clinical applications, and it may potentially be of use as part of the treatment protocol for snake envenomation.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Acuña-Castroviejo, D., Lopez, L.C., Escames, G., Lopez, A., Garcia, J.A., Reiter, R.J., 2011. Melatonin-mitochondria interplay in health and disease. Curr. Top. Med. Chem. 11, 221–240.

Acuña-Castroviejo, D., Escames, G., Venegas, C., Diaz-Casado, M.E., Lima-Cabello, E., Lopez, L.C., Rosales-Corral, S., Tan, D.X., Reiter, R.J., 2014. Extrapineal melatonin: sources, regulation, and potential functions. Cell. Mol. Life Sci.

Al, A.A., Al, M.K., Manthari, R.A., Khan, H.A., 2006. Time-course of lipid peroxidation in different organs of mice treated with *Echis pyramidum* snake venom. J. Biochem. Mol. Toxicol. 20, 93–95.

Ali, S.F., Tariq, M., Hasan, M., Haider, S.S., 1981. Effect of Russell's venom on lipid peroxidation in organs of the mouse. Toxicon 19, 903–905.

Antolin, I., Rodriguez, C., Sainz, R.M., Mayo, J.C., Uria, H., Kotler, M.L., Rodriguez-Colunga, M.J., Tolivia, D., Menendez-Pelaez, A., 1996. Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes. FASEB J. 10, 882–890.

Chen, S.T., Chuang, J.I., 1999. The antioxidant melatonin reduces cortical neuronal death after intrastriatal injection of kainate in the rat. Exp. Brain Res. 124, 241–247.

Cher, C.D., Armugam, A., Zhu, Y.Z., Jeyaseelan, K., 2005. Molecular basis of cardiotoxicity upon cobra envenomation. Cell. Mol. Life Sci. 62, 105–118.

Chippaux, J.P., 1998. Snake-bites: appraisal of the global situation. Bull. World Health Organ. 76, 515–524.

Crespo, E., Macias, M., Pozo, D., Escames, G., Martin, M., Vives, F., Guerrero, J.M., Acuña-Castroviejo, D., 1999. Melatonin inhibits expression of the inducible NO synthase II in liver and lung and prevents endotoxemia in lipopolysaccharide-induced multiple organ dysfunction syndrome in rats. FASEB J. 13, 1537–1546.

de Castro, I., Burdmann, E.A., Seguro, A.C., Yu, L., 2004a. Bothrops venom induces direct renal tubular injury: role for lipid peroxidation and prevention by antivenom. Toxicon 43, 833–839.

de Castro, I., Burdmann, E.A., Seguro, A.C., Yu, L., 2004b. Bothrops venom induces direct renal tubular injury: role for lipid peroxidation and prevention by antivenom. Toxicon 43, 833–839.

Deng, W.G., Tang, S.T., Tseng, H.P., Wu, K.K., 2006. Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding. Blood 108, 518–524.

Devi, C.M., Bai, M.V., Lal, A.V., Umashankar, P.R., Krishnan, L.K., 2002. An improved method for isolation of anti-viper venom antibodies from chicken egg yolk. J. Biochem. Biophys. Methods 51, 129–138.

El-Asmar, M.F., Farag, R.M., Shoukry, S., El-Smimi, I.R., 1979. Effect of scorpion (*Leiurus Quinquestriatus* H and E) venom on lipid metabolism. Toxicon 17, 279–283.

Escames, G., Acuña-Castroviejo, D., Lopez, L.C., Tan, D.X., Maldonado, M.D., Sanchez-Hidalgo, M., Leon, J., Reiter, R.J., 2006. Pharmacological utility of melatonin in the treatment of septic shock: experimental and clinical evidence. J. Pharm. Pharmacol. 58, 1153–1165.

Escames, G., Lopez, L.C., Garcia, J.A., Garcia-Corzo, L., Ortiz, F., Acuña-Castroviejo, D., 2012. Mitochondrial DNA and inflammatory diseases. Hum. Genet. 131, 161–173.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol. 186, 407–421.

Fry, B.G., 2005. From genome to venome: molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. Genome Res. 15, 403–420.

Galano, A., Tan, D.X., Reiter, R.J., 2011. Melatonin as a natural ally against oxidative stress: a physicochemical examination. J. Pineal Res. 51, 1–16.

Galano, A., 2013. On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. J. Pineal Res. 54, 245–257.

Garcia, J.J., Lopez-Pingarron, L., Almeida-Souza, P., Tres, A., Escudero, P., Garcia-Gil, F.A., Tan, D.X., Reiter, R.J., Ramirez, J.M., Bernal-Perez, M., 2014. Protective effects of melatonin in reducing oxidative stress and in preserving the fluidity of biological membranes: a review. J. Pineal Res. 56, 225–237.

Garcia-Corzo, L., Luna-Sanchez, M., Doerrier, C., Garcia, J.A., Guaras, A., Acin-Perez, R., Bullejos-Peregrin, J., Lopez, A., Escames, G., Enriquez, J.A., Acuña-Castroviejo, D., Lopez, L.C., 2013. Dysfunctional Coq9 protein causes predominant encephalomyopathy associated with CoQ deficiency. Hum. Mol. Genet. 22, 1233–1248.

Green, L.C., Ruiz de, L.K., Wagner, D.A., Rand, W., Istfan, N., Young, V.R., Tannenbaum, S.R., 1981. Nitrate biosynthesis in man. Proc. Natl. Acad. Sci. U. S. A. 78, 7764–7768.

Griffith, O.W., 1999. Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radic. Biol. Med. 27, 922–935.

Gutierrez, J.M., Theakston, R.D., Warrell, D.A., 2006. Confronting the neglected problem of snake bite envenoming: the need for a global partnership. PLoS. Med. 3, e150.

Hardeland, R., Tan, D.X., Reiter, R.J., 2009. Kynuramines, metabolites of melatonin and other indoles: the resurrection of an almost forgotten class of biogenic amines. J. Pineal Res. 47, 109–126.

Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74, 214–226.

Jaskot, R.H., Charlet, E.G., Grose, E.C., Grady, M.A., Roycroft, J.H., 1983. An automated analysis of glutathione peroxidase, S-transferase, and reductase activity in animal tissue. J. Anal. Toxicol. 7, 86–88.

Katkar, G.D., Shanmuga, S.M., Hemshekhar, M., Sharma, D.R., Sebastin, S.M., Sunitha, K., Rangappa, K.S., Girish, K.S., Kemparaju, K., 2014. Melatonin alleviates *Echis carinatus* venom-induced toxicities by modulating inflammatory mediators and oxidative stress. J. Pineal Res. 56, 295–312.

Khaldy, H., Escames, G., Leon, J., Bikjdaouene, L., Acuña-Castroviejo, D., 2003. Synergistic effects of melatonin and deprenyl against MPTP-induced mitochondrial damage and DA depletion. Neurobiol. Aging 24, 491–500.

Kolli, V.K., Abraham, P., Isaac, B., Kasthuri, N., 2013. Preclinical efficacy of melatonin to reduce methotrexate-induced oxidative stress and small intestinal damage in rats. Dig. Dis. Sci. 58, 959–969.

Konshina, A.G., Boldyrev, I.A., Utkin, Y.N., Omel'kov, A.V., Efremov, R.G., 2011. Snake cytotoxins bind to membranes via interactions with phosphatidylserine head groups of lipids. PLoS ONE 6, e19064.

Lerner, A.B., Case, J.D., Takahashi, Y., Lee, T.H., Mori, W., 1958. Isolation of melatonin, the pineal gland factor that lightens melanocytes. J. Am. Chem. Soc. 80, 2587.

Lowry, O.H., Rosebrough, H.N., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Marino, A., Di, P.R., Crisafulli, C., Mazzon, E., Morabito, R., Paterniti, I., Galuppo, M., Genovese, T., La, S.G., Cuzzocrea, S., 2009. Protective effect of melatonin against the inflammatory response elicited by crude venom from isolated nematocysts of *Pelagia noctiluca* (Cnidaria, Scyphozoa). J. Pineal Res. 47, 56–69.

Martin, M., Macias, M., Escames, G., Leon, J., Acuña-Castroviejo, D., 2000a. Melatonin but not vitamins C and E maintains glutathione homeostasis in t-butyl hydroperoxide-induced mitochondrial oxidative stress. FASEB J. 14, 1677–1679.

Martin, M., Macias, M., Escames, G., Reiter, R.J., Agapito, M.T., Ortiz, G.G., Acuña-Castroviejo, D., 2000b. Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo. J. Pineal Res. 28, 242–248.

Meier, J., Theakston, R.D., 1986. Approximate LD50 determinations of snake venoms using eight to ten experimental animals. Toxicon 24, 395–401.

Mohapatra, A.D., Kumar, S., Satapathy, A.K., Ravindran, B., 2011. Caspase dependent programmed cell death in developing embryos: a potential target for therapeutic intervention against pathogenic nematodes. PLoS Negl. Trop. Dis. 5, e1306.

Mukherjee, A.K., Maity, C.R., 1997. Effect of dietary supplementation of vitamin E in partial inhibition of Russell's viper venom phospholipase A2 induced hepatocellular and microsomal membrane damage in rats. Acta Physiol. Hung. 85, 367–374.

Mukherjee, A.K., Maity, C.R., 2002. Biochemical composition, lethality and pathophysiology of venom from two cobras? *Naja naja* and *N. kaouthia*. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 131, 125–132.

Poeggeler, B., Saarela, S., Reiter, R.J., Tan, D.X., Chen, L.D., Manchester, L.C., Barlow-Walden, L.R., 1994. Melatonin – a highly potent endogenous radical scavenger and electron donor: new aspects of the oxidation chemistry of this indole accessed in vitro. Ann. N. Y. Acad. Sci. 738, 419–420.

Reiter, R.J., Paredes, S.D., Manchester, L.C., Tan, D.X., 2009. Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. Crit. Rev. Biochem. Mol. Biol. 44, 175–200.

Rodriguez, C., Mayo, J.C., Sainz, R.M., Antolin, I., Herrera, F., Martin, V., Reiter, R.J., 2004. Regulation of antioxidant enzymes: a significant role for melatonin. J. Pineal Res. 36, 1–9.

Rodriguez, M.I., Carretero, M., Escames, G., Lopez, L.C., Maldonado, M.D., Tan, D.X., Reiter, R.J., Acuña-Castroviejo, D., 2007. Chronic melatonin treatment prevents age-dependent cardiac mitochondrial dysfunction in senescence-accelerated mice. Free Radic. Res. 41, 15–24.

Sewerynek, E., Abe, M., Reiter, R.J., Barlow-Walden, L.R., Chen, L., McCabe, T.J., Roman, L.J., Diaz-Lopez, B., 1995. Melatonin administration prevents lipopolysaccharide-induced oxidative damage in phenobarbital-treated animals. J. Cell Biochem. 58, 436–444.

Shashidharamurthy, R., Mahadeswaraswamy, Y.H., Ragupathi, L., Vishwanath, B.S., Kemparaju, K., 2010. Systemic pathological effects induced by cobra (*Naja naja*) venom from geographically distinct origins of Indian peninsula. Exp. Toxicol. Pathol. 62, 587–592.

Shvedova, A.A., Yanamala, N., Murray, A.R., Kisin, E.R., Khaliullin, T., Hatfield, M.K., Tkach, A.V., Krantz, Q.T., Nash, D., King, C., Ian, G.M., Gavett, S.H., 2013. Oxidative stress, inflammatory biomarkers, and toxicity in mouse lung and liver after inhalation exposure to 100% biodiesel or petroleum diesel emissions. J. Toxicol. Environ. Health A 76, 907–921.

Stefulj, J., Hortner, M., Ghosh, M., Schauenstein, K., Rinner, I., Wolfler, A., Semmler, J., Liebmann, P.M., 2001. Gene expression of the key enzymes of melatonin synthesis in extrapineal tissues of the rat. J. Pineal Res. 30, 243–247.

Tan, D.X., Manchester, L.C., Burkhardt, S., Sainz, R.M., Mayo, J.C., Kohen, R., Shohami, E., Huo, Y.S., Hardeland, R., Reiter, R.J., 2001. N1-acetyl-N2-formyl-5-methoxykynuramine, a biogenic amine and melatonin metabolite: functions as a potent antioxidant. FASEB J. 15, 2294–2296.

Tan, D.X., Manchester, L.C., Reiter, R.J., Plummer, B.F., Hardies, L.J., Weintraub, S.T., Vijayalaxmi, Shepherd, A.M., 1998. A novel melatonin metabolite: cyclic 3-hydroxymelatonin: a biomarker of in vivo hydroxyl radical generation. Biochem. Biophys. Res. Commun. 253, 614–620.

Tan, D.X., Manchester, L.C., Terron, M.P., Flores, L.J., Reiter, R.J., 2007. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? J. Pineal Res. 42, 28–42.

Venegas, C., Garcia, J.A., Escames, G., Ortiz, F., Lopez, A., Doerrier, C., Garcia-Corzo, L., Lopez, L.C., Reiter, R.J., Acuna-Castroviejo, D., 2012. Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations. J. Pineal Res. 52, 217–227.

Venkatesan, C., Sarathi, M., Balasubramanian, G., Thomas, J., Balachander, V., Babu, V.S., Bilal, S.M., Majeed, S.A., Madan, N., Raj, N.S., Vimal, S., Nambi, K., Hameed, A.S., 2014. Antivenom activity of triterpenoid (C34H68O2) from *Leucas aspera* Linn. against *Naja naja naja* venom induced toxicity: antioxidant and histological study in mice. Hum. Exp. Toxicol. 33, 336–359.

Wang, C.H., Wu, W.G., 2005. Amphiphilic beta-sheet cobra cardiotoxin targets mitochondria and disrupts its network. FEBS Lett. 579, 3169–3174.

Yap, M.K., Fung, S.Y., Tan, K.Y., Tan, N.H., 2014. Proteomic characterization of venom of the medically important Southeast Asian *Naja sumatrana* (equatorial spitting cobra). Acta Trop. 133C, 15–25.