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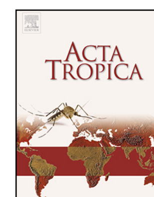

Melatonin inhibits snake venom and antivenom induced oxidative stress and augments treatment efficacy

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ABSTRACT

Snakebite is a neglected health hazard. Its patho-physiology has largely been focused on systemic and local toxicities; whereas, venom and antivenom induced oxidative stress has long been ignored. Antivenom therapy although neutralizes venom lethality and saves many lives, remains ineffective against oxidative stress. This prompted us to complement antivenom with an antioxidant molecule melatonin that would protect against oxidative stress and increase the efficacy of the existing snakebite therapy. Here we show that *D. russelli* and *E. carinatus* venoms induce strong oxidative stress that persists even after antivenom administration in mice model. Additionally, antivenoms also induce oxidative stress. Polyvalent antivenom induce more oxidative stress than monovalent antivenom. Strikingly, antivenom and melatonin together not only inhibit venom and antivenom induced oxidative stress but also significantly reduce the neutralizing antivenom dose. This study provides a therapeutic potential for enhancing the existing snakebite therapy. The combined treatment of antivenom + melatonin would prevent the upsurge of oxidative stress as well as minimize the antivenom load. Thus the investigation offers immense scope for physicians and toxinologists to reinvestigate, design new strategies and think beyond the conventional mode of antivenom therapy.

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1. Introduction

Globally about 5.5 million snakebite cases are reported annually, with death toll of 20,000 to 125,000 and 400,000 amputations. The most affected areas are the tropical and sub-tropical countries of Africa, south-east Asia, and Latin America (Kasturiratne et al., 2008; Warrell et al., 2013). Thus, World Health Organization in 2009 declared snakebite as a ‘neglected tropical disease’ (WHO, 2009). In India annually about 35,000 to 50,000 deaths are recorded (Warrell, 1999). Most of the bites in India are attributed to the ‘Big four’ snakes: *Naja naja* (cobra), *Bangarus caereleus* (krait), *Daboia russelii* (Russell’s viper) and *Echis carinatus* (saw-scaled viper). The bites of these snakes are well documented for various systemic toxicity and local tissue degradation (Herath et al., 2012; Jeevagan et al., 2013; Katkar et al., 2016; Perumal Samy et al., 2012; Torrez et al., 2014). Venom induced oxidative stress is another major concern of envenomation that has not been much focused in snakebite pathology. Oxidative stress impairs redox homeostasis and has been reported to cause hepato- and renal- toxicity, thrombocytopenia, methemoglobinemia and hypoxia (Abdel Moneim et al., 2015; Katkar et al., 2014; Santhosh et al., 2013a; Sharma et al., 2015).

Antivenom therapy is the only medically approved treatment against snake envenomation and employs the intravenous administration of monovalent or polyvalent antivenom. Though the therapy is effective against systemic toxicity, it remains ineffective against venom-induced local toxicity, inflammation and oxidative stress (Katkar et al., 2014; Girish and Kemparaju, 2011). The commercially available antivenoms in India are polyvalent in nature and produced against ‘big four’. The polyvalent antivenoms offer para-specific protection against many snake species and thereby do not need accurate diagnosis of the culprit snake, but they possess striking disadvantages too. They are often infused in large doses due to their lesser efficacies when compared to monovalent antivenoms (Arce et al., 2003; Shashidharamurthy and Kemparaju, 2007). This high dosage of antivenom leads to serum sickness and anaphy-

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laxis in susceptible individuals, which is treated by anti-histamine administration (Girish and Kemparaju, 2011; Ryan et al., 2015). As antivenom by itself is a pro-oxidant, its large dosage will further add on to the stress in victim's body. Therefore, apart from neutralizing systemic toxicities, it is also essential to neutralize the deleterious effects of oxidative stress that damage several vital organs. Thus, understanding the venom and as well as the antivenom induced oxidative stress and exploring ways to increase the efficacy of existing therapy is important.

Off late a large number of anti-oxidant molecules, to name a few: allopurinol, probenecid (Frezzatti and Silveira, 2011), melatonin (Katkar et al., 2014), lupeol derivatives (Katkar et al., 2015), crocein (isolated from *Crocus sativus*, Santhosh et al., 2013b), *N*-acetylcysteine, and *N*-acetylcysteine amide (Sunitha et al., 2013) have been screened for venom induced inhibition of oxidative stress. Of the anti-oxidant molecules studied, melatonin is the molecule of choice due to its high benefits. Melatonin (*N*-acetyl-5-methoxytryptamine) is a vital hormone produced in all vertebrates and exhibits functional diversity in various organisms (Reiter, 1991). It is the major secretory product of the pineal gland and is also produced in gut, lymphocytes, monocytes, bone marrow cells, ovary, lens of eyes, placenta etc. (Reiter et al., 2013; Reiter et al., 2014). Melatonin and its metabolites such as N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), N^1-acetyl-5-methoxykynuramine (AMK) and cyclic 3- hydroxymelatonin (C3-OHM) are best known for their free radical scavenging and antioxidative effects (Galano et al., 2013). Apart from its regulatory effect on circadian and seasonal rhythms, it also exhibits immunomodulatory, anti-inflammatory, antioxidant, anti-depressive and analgesic properties (Reiter et al., 2013). It has also been proposed to regulate cell proliferation (Reiter et al., 2000), apoptosis (Sainz et al., 2003), oxidative stress tolerance and inflammation in different experimental models (Rodriguez et al., 2004; Acuna et al., 2011). Its beneficial properties have been greatly explored in various patho-physiological conditions like cancer, cardiovascular diseases, inflammation, neurological disorders, diabetes, and hepatotoxicity to name a few (Bizzarri et al., 2013; Favero et al., 2014; Mauriz et al., 2013; Reiter et al., 2013). Melatonin has been documented to reduce the inflammatory response elicited by crude venom isolated from nematocysts of jellyfish, *Pelagia noctiluca* (Marino et al., 2009) and inhibit zinc-dependent matrix metalloproteinase-9 (Rudra et al., 2013). Also, melatonin administration has been demonstrated to attenuate *E. carinatus* venom induced inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-23, COX-2 and IL-10 and alleviated inflammatory pathological alterations in animal study (Katkar et al., 2014). It has also been reported to inhibit COX-2/iNOS and NF-κB/p300 signaling pathways (Yi et al., 2014). Based on these facts the present study is designed to investigate two parameters, firstly, the venom and antivenom induced deleterious oxidative stress that reduces the efficacy of the antivenom therapy and secondly, its efficient neutralization by supplementing melatonin with antivenom.

2. Materials and methods

2.1. Chemicals

Russell's viper (*Daboia russellii),* and saw-scaled viper (*Echis carinatus)* venoms were purchased from Irula Snake Catchers, Chennai, India. Commercial polyvalent equine anti-snake venom serums were purchased from Bharat Serums and Vaccines Ltd., Mumbai, India, (batch number A2513006) and Vins Bioproduct, Andra Pradesh, India, (batch number 01AS13089). Anti-equine goat IgG secondary antibody and molecular weight markers were obtained from Genei Bio products, Bangalore, India. Goat anti-rabbit IgG, 3,3′,5,5′-tetra methyl benzidine (TMB), agarose gel, immobilon-PVDF membranes, luminol, p-coumaric acid, Freund's complete and incomplete adjuvants, 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES), Dihydrodichlorofluorescein diacetate (DCFDA), ethylene diamine tetra acetic acid (EDTA), reduced glutathione (GSH), oxidized glutathione (GSSG), and O-phthalaldehyde (OPT), 1-chloro-2,4-dinitrobenzene (CDNB), were obtained from Sigma, St Louis, USA. Endochrome-K kit was purchased from Charles River Endosafe, Charleston, USA. Quercetin was purchased from Sisco Research Laboratories, Mumbai, India. Serum glutamic oxaloacetic transaminase (sGOT), serum glutamic pyruvate transaminase (sGPT), and creatinine commercial kits were purchased from AGAPEE Diagnostics Ltd. Kerala, India. Microwell plates, and pre-stained protein standard were procured from Thermo Fisher Scientific, Massachusetts, USA. All other chemicals used in this study were of analytical grade. Melatonin was dissolved in absolute ethanol and diluted in phosphate buffered saline (PBS), the amount of ethanol maintained <1%.

2.2. Experimental animals

Swiss albino mice (20–25 g) were collected from University Central Animal Facility, Department of Zoology, University of Mysore, Mysuru, India. New Zealand albino female rabbits 6 month old weighing 1.5–2.0 kg were obtained from Department of Livestock Production and Management, Veterinary College, Bengaluru, India, and housed under a controlled environment in University Central Animal Facility. All experiments were approved by the Institutional Animal Ethical Committee (UOM/IAEC/20/2012), Department of Studies in Zoology, University of Mysore, Mysuru, and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.3. Rabbit immunization and IgG purification

Immunization of rabbits and purification of antibodies were performed as described by Shashidharamurthy and Kemparaju (2007) with little modifications. *D. russelli* (*Dr*) venom/*E. carinatus* (*Ec*) venom (200 μg) in 100 μL PBS (10 mM phosphate buffered saline, pH 7.4) was thoroughly mixed with an equal volume of Freund's complete adjuvant and injected intra dermal to the back female rabbit at many sites. Further, 3 booster doses of venom were administered at the same concentration but with an equal volume of Freund's incomplete adjuvant at weekly intervals. Blood was drawn from marginal ear vein on 9th day after third booster dose and allowed to coagulate for 24 h at 8–10 °C to obtain the antiserum. Antiserum was subjected to ammonium sulfate precipitation to obtain crude immunoglobulin G (Ig G) fraction, which was then subjected to protein-A agarose column chromatography. Column was equilibrated with PBS and loaded with 5 mg crude Ig G fraction in 2 mL PBS, elution was carried out using 0.2 M glycine-HCl buffer, pH 2.9. Aliquots of 1 mL were collected and pooled after reading optical density at 280 nm and then neutralized using 1 M Tris–HCl buffer pH 8.0. Samples were further subjected to dialysis against PBS and used for the study. The monovalent antivenoms thus obtained were designated as *Dr*-IgG and *Ec*-IgG produced against *Dr* and *Ec* venoms respectively.

2.4. Endotoxin test (determination of LPS content)

Endotoxin test in antivenoms (BhAV, ViAV, *Dr*-IgG, *Ec*-IgG) was done using Endochrome-K kit. The test was carried out according to the manufacture's protocol. The amount of endotoxin level is expressed as endotoxin units per mL of antivenom protein (EU/mL).

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