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Melatonin elevates apoptosis in human platelets via ROS mediated mitochondrial damage

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ABSTRACT

Melatonin is a pineal hormone that regulates circadian and seasonal rhythms. The chronobiotic role of melatonin corresponds with a repertoire of pharmacological properties. Besides, it has a wide range of therapeutic applications. However, recent studies have demonstrated its direct interaction with platelets: at physiological concentration it promotes platelet aggregation; on the other hand, at pharmacological doses it raises intracellular Ca^{2+} leading to platelet activation, thrombus formation and cardiovascular disorders. In order to further probe its effects on platelets, the current study targeted platelet apoptosis and melatonin was found to stimulate apoptosis. The mitochondrial pathway of apoptosis was mainly investigated because of its susceptibility to oxidative stress-inducing factors including therapeutic and dietary elements. Melatonin significantly increased the generation of intracellular ROS and Ca^{2+}, facilitating mitochondrial membrane depolarization, cytochrome c release, caspase activation, protein phosphorylation and phosphatidylserine externalization. Further, the overall toxicity of melatonin on platelets was confirmed by MTT and lactate dehydrogenase assays. The elevated rate of platelet apoptosis has far reaching consequences including thrombocytopenia. Besides, platelets undergoing apoptosis release microparticles, which fuel thrombus formation and play a significant role in the pathophysiology of a number of diseases. In many parts of the world melatonin is an over-the-counter dietary supplement and alternative medicine. Since, melatonin displays platelet proapoptotic effect at a concentration attainable through therapeutic dosage, the present study sends a warning signal to the chronic use of melatonin as a therapeutic drug and questions its availability without a medical prescription.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is an omnipresent indoleamine in both plant and animal kingdoms including insects, arthropods, planarians and molluscs [1]. In humans, it is an endogenous antioxidant produced by the pineal gland and regulates circadian and seasonal rhythms, and helps in the regulation of other hormones. The chronobiotic role of melatonin corresponds with the pharmacological properties including immunomodulatory, anti-inflammatory, antioxidative, anxiolytic, antidepressive, sedative and analgesic [2–3]. Various studies have reported the therapeutic properties of melatonin against neurodegenerative disorders, diabetes, hepatotoxicity, cancer, and radiation induced complications [4–5]. However, there are also studies that have reported its pro-oxidative properties, which depend on its concentration as well as the components of the interacting cells. Its pro-oxidant effect on human erythrocytes under the conditions of accelerated damage generated during prolonged incubation has also been demonstrated [6].

In humans, melatonin is reported to regulate various important neuroendocrine and physiological functions [7]. It can enter subcellular compartments and probably bind to few cytosolic proteins. The signalling of melatonin is exerted through G-protein coupled receptor or nuclear receptor activation [7–8]. The receptors have been identified in a various tissues such as brain, ovary, teeth and bone. Melatonin binding sites have also been characterized in cells including retina, blood lymphocytes, manocytes and platelets [4,7].

Platelets are megakaryocyte-derived anuclear cells and their role in hemostasis, thombosis and wound healing is well-established [9]. However, variations in platelet number and function result in bleeding disorders and thombotic diseases [10]. Up-regulated platelet activation may result in arterial thombosis. Altered platelet functions are also the root cause of the pathophysiology of

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multifactorial diseases including coronary heart disease (CHD) and other cardiovascular diseases (CVDs) [10]. Besides, platelets release a wide spectrum of biologically active substances like growth factors and pro-inflammatory molecules and thereby, mediate inflammatory and immune reactions. Apart from promoting coagulation, they are key mediators in fibrinolysis and angiogenesis [11].

Several studies have reported the existence of direct effects of melatonin on platelets including aggregation, and release of ATP and serotonin [12]. Melatonin at physiological level (<1 μM) stimulates platelet aggregation, whereas at concentration >10 μM, it inhibits collagen/ADP/epinephrine-induced aggregation. In addition, at higher concentrations, melatonin inhibits platelet cyclooxygenase and reduces arachidonic acid-induced aggregation and thomboxane B2 production [13]. Agonist-induced platelet aggregation involves the activation of various intracellular signalling including Ca^{2+} mobilization. In addition, reactive oxygen species (ROS) also alter Ca^{2+} signalling [7,12]. It is reported that melatonin antagonizes the binding of Ca^{2+} to calmodulin, a calcium-binding receptor protein that regulates the contractile properties of platelets. Further, a recent study showed the alteration of Ca^{2+} levels in platelets by inositol 1,4,5-triphosphate-independent mechanism [12].

Melatonin supplements help in the management of jet lag, insomnia, blood pressure, daytime fatigue, ovarian physiology and fibromyalgia [12]. In addition, melatonin containing cosmetics may protect the skin against sunburn, premature aging and damages [7,14]. Although, melatonin has gained importance as a therapeutic molecule, the possible adverse effects have been ignored to some extent. In the present study, we report for the first time the pharmacotoxicological effect of melatonin in human platelets, in particular apoptosis. The apoptotic events in platelet apoptosis include the increased production and release of ROS, particularly hydrogen peroxide (H_2O_2), depolarization of mitochondrial inner transmembrane potential ($\Delta\Psi m$), release of apoptotic factors and phosphatidylserine (PS) externalization.

2. Materials and methods

2.1. Chemicals/ Reagents

Calcium ionophore (A23187), benzamidine hydrochloride, *N*-acetyl-Leu-Glu-His-Asp trifluoro methylcoumarin (AC-LEHD-FMC), acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC), glutaraldehyde, sodium orthovanadate (Na_3VO_4) Dimethyl sulfoxide (DMSO), fluorescein isothiocyanate (FITC)-labeled annexin V, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetylester (CM-H2DCFDA), 5,5′-6,6′-tetrachloro-1, 1′,3,3′-tetraethyl benzimidazolylcarbocyanine iodide (JC-1), leupeptin hydrochoride, N-(2-Hydroxyethyl) piperazine-N′-ethanesulfonic acid (HEPES), fura-2/AM, monoclonal anti-phosphotyrosine antibody and dithiotheitol (DTT), were from Sigma Chemicals (USA). Monoclonal anti-cytochome c antibody was from Epitomics (USA). Anti-Caspase-3 antibody was from Santa Cruz Biotechnology (USA). Collagen type-I was from Crono-log Corporation (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from HiMedia Laboratories (India). LDH kit was from Siemens Autopak (India). Melatonin and homovanillic acid (HVA) were from SRL (India). All other reagents were of analytical grade.

2.2. Preparation of platelet-rich plasma and washed platelets

Venous blood was drawn from healthy drug-free human volunteers (non-smokers) with informed consent as per the guidelines of Institutional Human Ethical Committee, University of Mysore, Mysore. Platelet-rich plasma (PRP) and washed platelets (WP) suspensions were prepared of Kumar et al. [15]. The cell count was determined in both PRP and WP suspension using a Neubauer chamber and adjusted to 5×10^8 cells/ml in the final suspension using Tyrode's albumin buffer (pH 7.4).

2.3. Determination of endogenously generated reactive oxygen species and H_2O_2

Endogenous ROS and H_2O_2 production in platelets was determined according to the method of Thushara et al. [16], with slight modifications. PRP and WP suspension were taken separately in 96-well microtiter plates and treated with calcium ionophore A23187 (2 μM) as positive control or melatonin in increasing doses (0–50 μM), final volume was made up to 200 μL with HEPES-buffered saline (HBS). The control (untreated) and treated platelets were then incubated with 10 μM CMH2DCFDA (for ROS) or with 100 μM HVA (for H_2O_2) for 30 min at 37 °C, fluorescence was recorded using a Varioskan multimode plate reader (Thermo Scientifics, USA.).

2.4. Estimation of intracellular calcium

Intracellular Ca^{2+} concentration was measured in PRP and WPs with slight modifications according to the method of Thushara et al. [16]. Both PRP and WPs, taken in 96-well microtiter plates were treated with A23187 (2 μM) or melatonin in increasing doses (0–100 μM) and the final volume made up to 200 μL with modified Tyrode's solution (pH 7.4) incubated for 1 h followed by incubation with 2 μM fura-2/AM.

2.5. Determination of changes in mitochondrial membrane potential ($\Delta\Psi m$)

The Cationic dye JC-1 was used to detect changes in the $\Delta\Psi m$ according to the method of Thushara et al. [16]. Both PRP and WPs, independently taken in 96-well microtiter plates were treated as described in the previous section. The samples were then loaded with JC-1 (2 μM).

2.6. Detection of cytochrome c release

Cytochome c (cyt.c) release was detected by immunoblotting. The WPs were independently processed as discussed previously. Cells were then frozen for 30 min at −20 °C and kept for 10 min at room temperature for thawing. The cycle was repeated for 4 times to get platelet lysate. Cytosolic and cytoskeleton fractions were separated by centrifugation at 16000*g* for 15 min. Cytosolic proteins were separated by 10% SDS–PAGE and electrophoretically transferred on to PVDF membrane. Blots were then incubated overnight with 10% BSA in tris-buffered saline with 0.1% tween 20 (TBST). Membranes were incubated with anti-cytochome c antibody (1:1000) in TBST for 2 h. Blots were then developed by enhanced chemiluminescence method [16].

2.7. Caspase assay

PRP and WPs were independently processed as discussed previously. Platelet lysate was prepared was prepared according to the method of Thushara et al. [16]. The pellet is the cytoskeleton-rich (Triton-insoluble) fraction, which was subjected to caspase activity.

2.8. Determination of PS externalization

PRP and WPs were independently processed as discussed previously. PS exposure was determined according to the method of

Thushara et al. [16]. Cell staining was measured in a multimode plate reader by exciting the samples at 496 nm and emission was recorded at 560 nm.

2.9. Detection of protein tyrosine phosphorylation and caspase-3

For protein tyrosine phosphorylation, WPs were stimulated independently by incubating with collagen (1 μg/ml) or melatonin in increasing concentration (0–100 μM) for 10 min. For caspase-3 expression, WPs were treated independently with calcium ionophore A23187 (2 μM) as positive control or melatonin in increasing concentration (0–100 μM) for 1 h. Further, the protein expression was determined according to the method of Kumar et al. [15].

2.10. MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was performed to assess the cell viability. PRP was taken separately in 96-well microtiter plates and treated with different doses of either A23187 (0–10 μM) or melatonin (0–1000 μM) and final volume was made up to 200 μL with HBS. After 1 h of incubation, 250 μM of MTT was added and incubated for additional 3 h. Thereafter, MTT was removed and remaining formazan crystals were completely dissolved in DMSO. The absorbance was recorded at 570 nm [17].

2.11. Measurement of LDH leakage

PRP was treated either with different doses of melatonin (0–500 μM) or with calcium ionophore A23187 (2 μM) for 1 h. After centrifugation, platelets were pelleted by centrifugation at 800*g* for 10 min. Supernatants were used to detect LDH release by using Siemens autopak LDH kit, according to the manufacturer's protocol. The assay was performed in a time course of decrease in NADH absorbance at 340 nm for 0–1 min.

2.12. Statistical analysis

Results were expressed as mean ± SEM of five independent experiments. Statistical significance among groups was determined by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means [$n = 5$, $p < 0.05$ (*)].

3. Results

3.1. Effect of melatonin on ROS and H_2O_2 generation

In order to evaluate the effect of melatonin on platelets, oxidative stress mediators such as ROS and H_2O_2 were estimated. The A23187-induced levels of ROS and H_2O_2 were considered as 100% induction. About 1.72 and 2.90 fold increased ROS was observed in A23187-treated PRP and WPs, respectively compared to control. In contrast, melatonin induced ROS generation in both PRP and WPs dose-dependently. At 20 μM, melatonin significantly induced generation of ROS in WPs compared to control and found to be more potent than A23187 (Fig. 1A). Further, 2.92 and 3.0 fold increased generation of H_2O_2 was observed in A23187-treated PRP and WPs, respectively. A dose-dependent generation of H_2O_2 was observed in WPs treated with melatonin. At 100 μM concentration, significant increase in H_2O_2 generation with 89.7% induction was observed (Fig. 1B)

3.2. Effect of melatonin on intracellular calcium levels

The A23187-induced level of intracellular Ca^{2+} was considered as 100% induction. A23187 treatment caused 1.35 and 1.66 fold in-

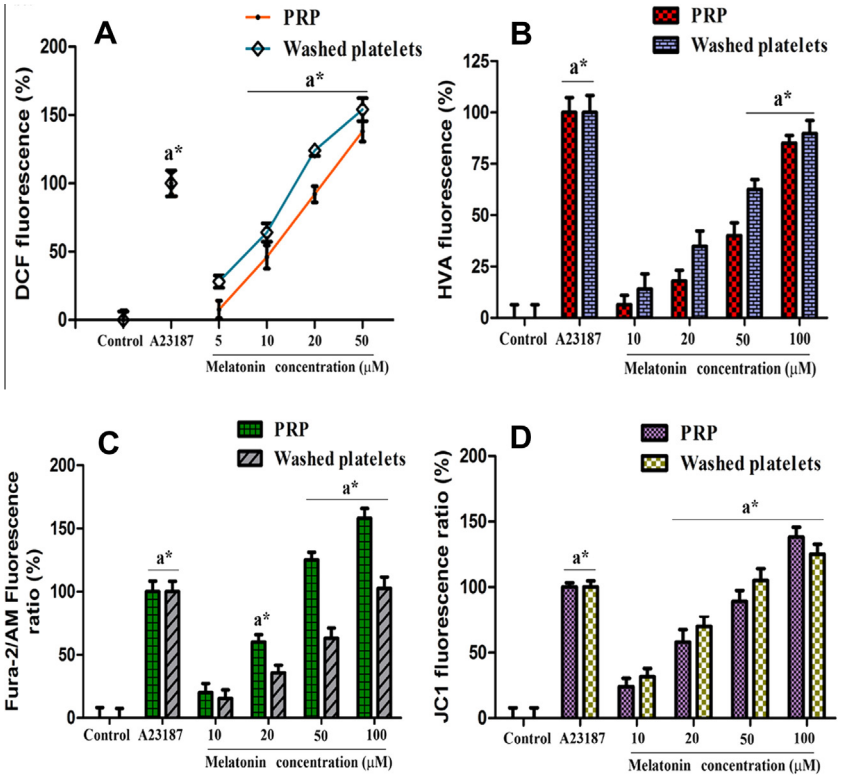

Fig. 1. Melatonin induced (A) ROS (B) H_2O_2 generation, (C) intracellular calcium and (D) mitochondrial membrane depolarization in PRP and WPs. Values are presented as means ± SEM ($n = 5$), expressed as fold increase in DCF fluorescence (for ROS), HVA fluorescence (for H_2O_2), percentage increase in Fura-2AM fluorescence (intracellular calcium) and percentage increase in JC-1 fluorescence ratio ($\Delta\Psi m$) relative to control. a*: significant compared to control ($p < 0.05$).

crease in the intracellular calcium levels compared to control in both PRP and WPs, respectively (Fig. 1C). Melatonin dose-dependently increased intracellular Ca^{2+} in both PRP and WPs. At 50 μM, there was significant increase in Ca^{2+} levels in both PRP and in WPs compared to control. Melatonin at higher dose of 100 μM was found to be more potent in increasing Ca^{2+} compared to A23187.

3.3. Dissipation of $\Delta\Psi m$ by melatonin

The fold decrease in $\Delta\Psi m$ potential was considered as 100% dissipation of membrane potential. Treatment of platelets with A23187 caused 1.46 and 1.54 fold decrease in $\Delta\Psi m$ potential both in PRP and WPs compared to resting platelets (Fig. 1D). Melatonin dose-dependently induced dissipation of $\Delta\Psi m$ in both PRP and WPs. At 20 μM, it significant induced membrane depolarization compared to control with 58% and 70% in PRP and WPs, respectively. At 100 μM, melatonin was found to be more potent in dissipating $\Delta\Psi m$ than A23187.

3.4. Cytosolic cytochome c expression

A23187 treatment caused release of cyt. c into cytosol. Additionally, melatonin dose-dependently increased the expression of cyt.c in cytosol. At 50 μM concentration melatonin was found to be highly efficient than A23187 in releasing cyt.c into cytosol (Fig. 2A).

3.5. Caspase activity

Treatment of platelets with A23187 caused 2.32 and 2.28 fold increase in caspase-9 activity in both PRP and WPs, respectively compared to control. Caspase activation caused by A23187 was considered as 100% increase in the activity. Additionally, melatonin dose-dependently activated caspase-9 starting from 10 μM. At 100 μM, melatonin triggered significant increase in the caspase-9 activity (Fig. 3A).

Treatment of both PRP and WPs with A23187 caused 1.54 and 1.98 fold increase in the caspase-3 activity. Melatonin dose-dependently activated caspase-3 and significant increase in the activity was observed at 100 μM concentration (Fig. 3B). Activation of caspase-3 was once again confirmed by immunoblot, wherein melatonin (100 μM) induced significant increase in the active form of caspase-3 (Fig. 3C).

3.6. Effect of melatonin on PS scrambling

Platelet treatment with A23187 induced 1.42 and 1.56 fold increase of PS externalization in PRP and WPs, respectively. Melatonin being found to activate previously discussed apoptotic markers also manifested PS scrambling in a dose-dependent manner. Significant induction was observed at 20 μM with 77% PS scrambling compared to control in PRP. At 100 μM, melatonin was found to be more potent than A23187 in inducing PS externalization (Fig. 2B).

3.7. Determination of protein phosphorylation

In order to evaluate the effect of melatonin on intracellular signalling pathway, tyrosine phosphorylation was evaluated. Resting platelets were found to have basal level of tyrosine kinase activity. However, significant increase in the activation of tyrosine kinases was observed in collagen-treated platelets. Similarly, there was dose-dependent increase in the intensity of the bands of melatonin-treated platelets, suggesting increased tyrosine kinase activity. At 100 μM, melatonin stimulated significant increase in the phosphorylated proteins involved in platelet apoptosis (Fig. 3D).

3.8. Cytotoxic effect of melatonin

There was significant reduction in the number of viable platelets to 50% when treated with A23187 (2 μM). Melatonin at concentration increasing from 100 μM significantly decreased MTT reduction activity suggesting its cytotoxic nature (Fig. 4A). Further LDH leakage was also analysed. There was a significant release of LDH into the medium by the platelets treated with melatonin (100 μM), confirming its cytotoxic effect (Fig. 4B).

4. Discussion

In spite of being a crucial pineal hormone, which is therapeutically applicable as an antioxidant or as an orchestrator of immunological, cellular and endocrine responses, recent studies have demonstrated the direct interaction of melatonin with platelets. At physiological concentration (<1 μM) it was found to promote platelet aggregation; conversely, at concentrations <10 μM, it was found to inhibit agonist (collagen, ADP and epinephrine)-induced aggregation via cyclooxygenase pathway. Besides, it has been demonstrated that pharmacological dose of melatonin (500 μM) raises intracellular free Ca^{2+} in platelets, which leads to platelet activation, thrombus formation and cardiovascular disorders [12,18]. In order to further explore its effects on platelets, the current study targeted platelet apoptosis. With the results achieved in the

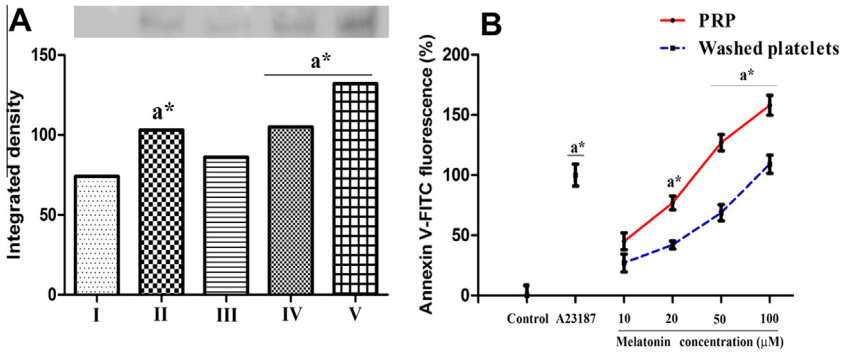

Fig. 2. Melatonin induced (A) release of cytosolic cytochrome C and (B) PS externalization in PRP and WPs. (A)*Lane 1*- resting platelets (untreated). *Lane 2*- platelets treated with A23187 (2 μM). *Lanes 3, 4 and 5*- platelets treated with 10, 50 and 100 μM of melatonin, respectively. Histograms represent the expression levels of cyt.c in respective groups. (B) Values are presented as means ± SEM ($n = 5$), expressed as fold increase in Annexin-V FITC fluorescence ratio. a*: significant compared to control ($p < 0.05$).

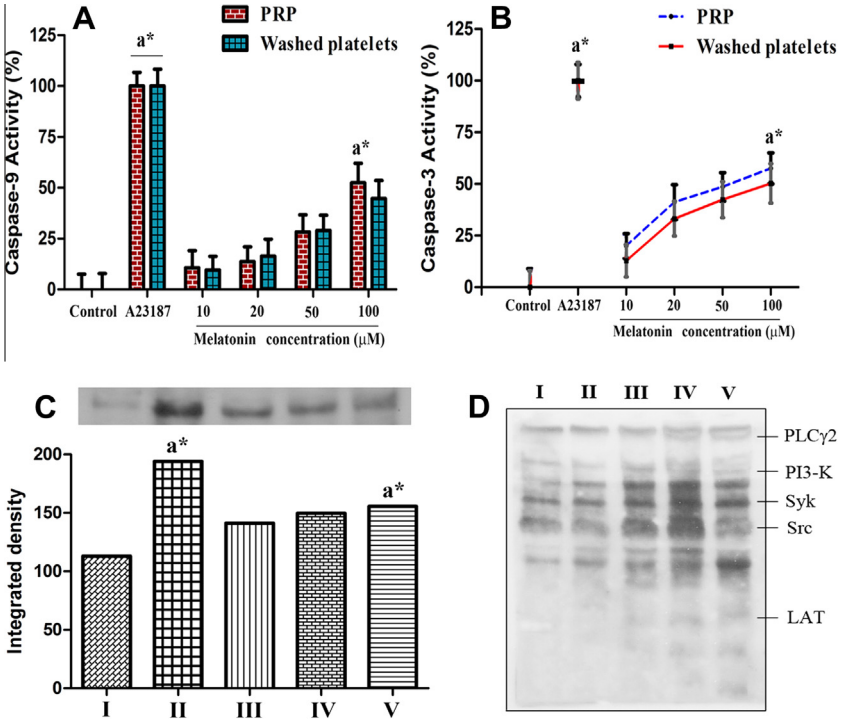

Fig. 3. Melatonin induced (A) caspase-9 and (B) caspase-3 activities (C) expression levels of caspase-3 and (D) protein phosphorylation in platelets. (A & B) Values are presented as mean ± SEM ($n = 5$), expressed as percentage increase in caspase activity relative to control. a*: Significant compared to control ($p < 0.05$). (C)*Lane 1* – resting platelets (untreated). *Lane 2* – platelets treated with A23187 (2 μM). *Lanes 3, 4 and 5* – platelets treated with 10, 50 and 100 μM of melatonin, respectively. Histograms represent the expression levels of caspase-3 in respective groups. (D) Lane 1– resting platelets (untreated). Lane 2, 3 and 4 – platelets treated with 10, 50 and 100 μM concentration of melatonin, respectively. Lane 5 – platelets treated with type I collagen.

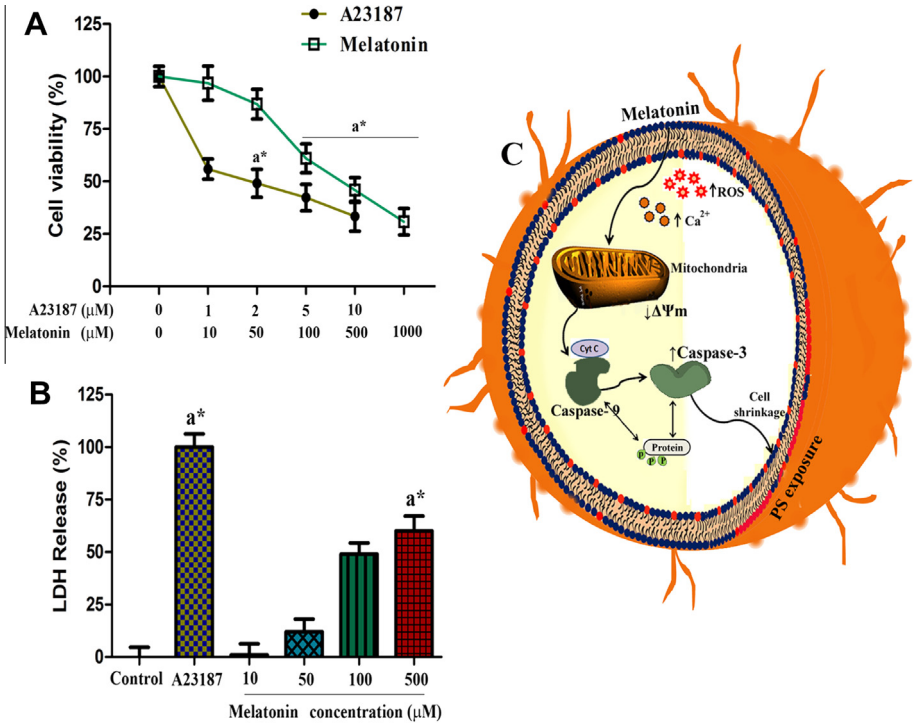

Fig. 4. Cytotoxic effects of melatonin: (A) *MTT assay:* PRP was treated independently with varying concentration of melatonin (0–1000 μM) and A23187 (0–10 μM). (B) *Lactate dehydrogenase release assay:* PRP was treated independently with A23187 (2 μM) and varying concentration of melatonin (0–500 μM). Values are presented as mean ± SEM ($n = 5$). a*: Significant compared to control ($p < 0.05$). (C) A schematic representation depicting the proposed mechanism for melatonin induced platelet apoptosis.

current study, melatonin was found to trigger platelet apoptosis at concentration range 10–100 μM. The mitochondrial/intrinsic pathway of apoptosis was mainly probed because of its susceptibility to oxidative stress-inducing factors such as, tumor suppressor

genes, therapeutic and dietary elements, which may alter the mitochondrial membrane integrity.

Melatonin has been shown to be as effective as glutathione and vitamin E in scavenging hydroxyl radicals and thereby, protects the cells from damage by ROS [19]. It also shows pro-oxidative effect sometimes, which might be accountable for its anti-microbial properties. As evident from the present study, melatonin induces a significant generation of ROS, particularly H_2O_2 in platelets. Studies show that H_2O_2 is the principal ROS that initiates the apoptotic events in platelets though the mitochondrial pathway by altering the $\Psi\Delta m$. Thereby, to assess the influence of melatonin on platelet mitochondria, the $\Psi\Delta m$ was measured. In addition to their primary role in the cellular energy metabolism, the mitochondria also play a critical role in the intrinsic pathway of apoptosis. Mitochondria are the key targets of cellular oxidative stress, which hampers the electron transport chain, in due course leading to an amplification of ROS generation and mitochondrial permeability transition pore (MPTP) formation. This results in the depolarization of $\Psi\Delta m$ [10]. The current results demonstrate that melatonin considerably evokes $\Delta\Psi m$ depolarization. It is indicative of the lethal effects of melatonin on mitochondria, thereby facilitating the ensuing events of apoptosis.

Elevated level of intracellular Ca^{2+} is one of the major contributing factors towards the changes in $\Delta\Psi m$ as well as MPTP formation [10]. Therefore, the effect of melatonin on intracellular Ca^{2+} was scrutinized to further ascertain its potentiality in inducing $\Delta\Psi$m depolarization. It was found to elicit a significant increase in intracellular Ca^{2+} concentration. Generally, several agonists of platelets elevate cytosolic Ca^{2+} either by triggering its release from intracellular stores or by the efflux of extracellular Ca^{2+} though plasma membrane. Of late, melatonin was reported to trigger platelet intracellular Ca^{2+} through depletion of mitochondrial Ca^{2+} stores and store-operated calcium entry (SOCE). The process of SOCE is actually controlled by the concentration of Ca^{2+} in platelet dense tubular system (DTS), which is also known as an analogue to sarcoplasmic reticulum or endoplasmic reticulum. Further, it was also shown that melatonin directly interacts with Ca^{2+} binding intracellular proteins like calmodulin, calreticulin/tubulin thereby antagonizes the Ca^{2+} binding to calmodulin allowing drastic rise in Ca^{2+}. In addition, intracellular Ca^{2+} levels also play an essential role in platelet activation by regulating the transient receptor potential channels (TRPC). The structure of all identified mammalian TRPC channels show C-terminal calmodulin and inositol 1,4,5-trisphosphate receptors (IP3Rs)-binding (CIRB) site. Calmodulin has been reported to induce Ca^{2+}-dependent feedback inactivation of TRPC channels through the interaction with the TRPC C-terminal calmodulin- and IP3Rs-binding (CIRB) site [7,12,20].

Loss of $\Psi\Delta m$, and MPTP formation lead to increased permeability of inner and outer mitochondrial membranes, thus facilitating the release of proapoptotic proteins from mitochondria to cytosol. The elevated levels of mitochondrial proteins in the cytosol are considered as reliable markers to identify an apoptotic cell [21]. The immunoblot of cytosolic cyt.c in melatonin-treated platelets obviously indicates the dose-dependent increase in the protein concentration. The cytosolic cyt.c associates with Apaf 1 and procaspase-9 to form the apoptosome complex, following which procaspase-9 self-activates to caspase-9, the initiator caspase of the intrinsic pathway. Caspases, the class of cysteine-dependent aspartases which slice proteins at aspartate residues, hold a fundamental responsibility in the apoptotic death of a cell. The activated procaspase-9 in turn triggers the activation of procaspase-3 to caspase-3, the executioner caspase that brings about the disintegration of cytoskeletal proteins, particularly gelsolin which is its main substrate. This event causes morphological modifications such as, the formation of filapodia and apoptotic bodies in the apoptotic platelets [18]. Melatonin was found to stimulate the activation of caspases-9 and -3 in a dose-dependent manner, which further confirms the pro-apoptotic effects of melatonin in platelets. Besides, crosstalk between proteolytic cleavage and phosphorylation in apoptosis and other cellular processes has long been reported. Very recently, it was elucidated that caspase cleavage can expose new sites on proteins for phosphorylation and similarly, apoptosis targeted phosphorylation of caspase recognition sites (P3 position) can endorse the cleavage of proteins. In addition, caspase cleavage also activates kinases such as DNA-PK, which contributes to the formation of apoptotic-specific phosphorylation network in a cell [20]. Further, the present study also demonstrated that melatonin was capable of provoking PS externalization, an indispensable biochemical feature of an apoptotic cell, which signals the phagocytic cells, finally leading to cell death. The overall toxic effects of melatonin towards platelets were further confirmed by the positive results for MTT and elevated LDH.

Altogether, the findings of the present study suggest the platelet proapoptotic nature of melatonin (Fig. 4C). The proapoptotic property of melatonin has previously been demonstrated in tumor cells. However, accelerated rate of platelet apoptosis has far reaching consequences. For instance, it might lead to a drop in platelet count, a condition referred to as thrombocytopenia, which leads to a number bleeding disorders and delay in wound healing. Besides, platelets undergoing apoptosis release negatively charged PS-positive plasma membrane vesicles, which are known as microparticles (MPs). MPs provide effective surface area for fibrin deposition and assembly of coagulation factors, and thus fuel thrombus formation. Circulating MPs are also pro-inflammatory in nature and are capable of exacerbating coagulation and influence vascular functions as well. These events set the stage for the development of thombotic disorders, CVDs and inflammatory arthitis. Thus, the MPs generated from platelets play a significant role in the pathophysiology of a number of diseases [22]. In many parts of the world melatonin is counted as a dietary supplement and is an easily available over-the-counter alternative medicine. Moreover, melatonin exhibits platelet proapoptotic effect well within the concentration achievable though therapeutic dosage. In this background, the present study sends a warning signal to chronic use of melatonin as a therapeutic drug. Recently, therapeutic phytochemicals like crocin and cinnamtannin B1 have been claimed to be platelet protective against oxidative stress-induced platelet apoptosis [16], which can also be included in the melatonin pharmacology as an auxiliary therapy.

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