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ORIGINAL PAPER

Impact of melatonin on platelets during oxidative stress – an in vitro approach

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ABSTRACT

Introduction and aim. Platelets are susceptible to oxidative damage due to metabolic pathways and oxygen-rich environments. Antioxidants combat oxidative stress (OS) and are currently employed in therapeutics. Melatonin has potent antioxidant properties; however, it has not been explored in platelet OS models. This study investigates the effect of melatonin on platelets during 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced OS.

Material and methods. Platelets from Wistar rats (n=5) were grouped into controls (untreated), free radical-inducer (FRI: AAPH-treated), melatonin-treated (AO), and preincubated with melatonin and AAPH-treated (FRI+AO). OS and platelet markers were analyzed.

Results. Antioxidant defenses decreased in FRI, whereas increased in AO and FRI+AO. Lipid peroxidation (LPO) increased in FRI, whereas advanced oxidation protein products (AOPP) and metabolism increased in AO compared to controls. Superoxides, AOPP, and ATP secretion increased, whereas LPO decreased in FRI+AO compared to FRI. However, aggregation increased in FRI and AO compared to Controls, whereas decreased in FRI+AO compared to FRI.

Conclusion. OS models can give insights into the underlying redox status of the cells and modulations of antioxidants in platelets. The findings indicate that melatonin can modulate antioxidant defenses and alleviate OS in platelets. This study lays the foundation for further in vivo studies on platelet pathophysiology.

Keywords. antioxidants, melatonin, oxidative stress, platelets

Introduction

Oxidative stress (OS) is a primary causative factor that disrupts hemostasis and can potentially have pathological consequences. Reactive species can affect redox signaling and lead to cellular damage. These radicals damage the membrane by lipid peroxidation and also cause protein oxidation, eventually altering the functioning of proteins.[1] OS contributes to pathophysiological conditions like Alzheimer's, Parkinson's, cardiovascular diseases, and cancer.[2] Therefore, OS models simulate physiological conditions comparable to infections, diseases, stress, and drug toxicity. They can provide insights into the underlying redox mechanisms and their interaction with endogenous defenses.[3] 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) which can simulate the OS conditions in vitro.[4] It undergoes homolytic cleavage to form two carbon-centered radicals ($R^{\bullet}$) which produce peroxyl radicals in the presence of oxygen. These highly reactive radicals can initiate chain reactions, thereby rendering proteins and polyunsaturated fatty acids extremely prone to oxidative damage.[5]

Platelets are anucleate cells with a crucial role in hemostasis. Physiological processes in platelets contin-

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uously generate reactive oxygen species (ROS). ROS generation can overwhelm the endogenous antioxidant machinery and result in a redox imbalance. OS in platelets can lead to excessive activation, malfunction, or destruction.[6] This increases the risk of platelet-related disorders, such as thrombotic disorders and thrombocytopenia. OS can also shorten the lifespan of platelets by triggering apoptotic pathways.

However, antioxidants can enhance the antioxidant defenses in platelets. Vitamin C, vanillic acid, L-carnitine, coumaric acid, and other antioxidants have been studied for their beneficial effects on platelets.[7] Melatonin can penetrate the cell membrane to neutralize endogenous hydroxyl radicals and stimulate glutathione peroxidase.[8] In vitro studies have determined that 1 mmol/L melatonin can attenuate oxidative damage in different cell types.[9-12] Melatonin has proven beneficial in reducing lipid peroxidation and regulating intracellular calcium levels in platelets.[12-14] Although the effect of melatonin varies in physiological and pharmacological conditions, its role as an antioxidant has been widely studied.[15] However, its overall effect on oxidative stress, metabolism, and functions in platelets is unclear.

Melatonin can be a promising antioxidant due to its protective effects. It can prevent oxidative damage, stimulate platelet production, and enhance platelet function.[16-17] Melatonin supplementation can potentially modulate the OS situation and be beneficial in platelet disorders.

The rationale of this study is to investigate the effects of melatonin for potential therapeutic applications in platelet disorders. Studies indicate that melatonin promotes platelet production, attenuates oxidative damage, and prevents platelet apoptosis. Hence, melatonin could be a potential alternative therapeutic tool.

Aim

It is imperative to study OS in platelets to understand the underlying mechanisms using antioxidants for their targeted actions. This is the first study to elucidate the responses of platelets to melatonin in free radical-induced OS conditions, especially the interactions of peroxyl radicals with melatonin in platelets.

Material and methods

Reagents

2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), melatonin, Epinephrine, bathocuproine disulfonic acid disodium salt, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and bovine serum albumin were purchased from Sigma-Aldrich Chemicals (Bengaluru, India). Cytochrome C, β-Nicotinamide adenine dinucleotide disodium salt and collagen (ex. Marine Fish extrapure) was purchased from SRL Chem (Mumbai, India). Glucose reagent kit (GOD-POD method; Catalogue no. GLU-L-D-1153) was from Aspen Laboratories (Delhi, India).

Methods

Male Wistar rats (160–180 g) procured from Bioally Animal Facility and Research Pvt. Ltd. (Bengaluru, India) were used for this study. Experimental study was conducted according to the Institutional Ethical Committee regulations (1810/PO/RcBizbt/S/15/CCSEA).

The physiology of Wistar rats is similar to humans. These animals are bred and maintained in controlled laboratory conditions and exhibit minimal variations. Hence, they are valuable models for toxicological studies, and the outcomes can be directly related to experimental interventions.

Experimental design

The platelets (20 *Wistar* rats, n=5) were grouped as follows: (i) controls (untreated platelets); (ii) free radical initiator (FRI) (2 mmol/L AAPH for 30 min); (iii) antioxidant (AO) (1 mmol/L melatonin for 60 min); and (iv) FRI+AO (1 mmol/L melatonin for 60 min followed by 2 mmol/L AAPH for 30 min).

The samples were gently resuspended in Tyrode's buffer (pH 7.4) and stored at 22°C until analysis. These platelet samples were aliquoted in aseptic conditions and assessed for antioxidant defense, OS markers, and platelet functions.

Sample preparation

Blood collection

Male Wistar rats were mildly anesthetized, and blood was collected into polypropylene tubes containing CPDA-1 anticoagulant (citrate phosphate dextrose adenine).[18]

Isolation of platelets

Platelets were isolated from whole blood according to Carneiro et al.[19] Briefly, 8 mL of whole blood was spun at 1500 rpm at 22°C for 15 min. The supernatant containing platelet-rich plasma (PRP) was further centrifuged at 4000 rpm at 22°C for 15 min. The pellet containing platelets was resuspended in Tyrode's buffer.

Antioxidant defenses

Superoxide dismutase (SOD)

Platelet samples (100 µL) were mixed with 880 µL of 0.05 M carbonate buffer (pH 10.2 with 0.1 mmol/L ethylenediamine tetraaceticacid). 20 µL of 30 mmol/L epinephrine was added to it. The absorbance was read immediately at 480 nm for 4 min. The amount of enzyme that inhibits the oxidation of epinephrine by 50% is equal to 1 unit of SOD.[20]

Catalase
10 µL of absolute ethanol was added to 100 µL of the platelet sample. The mixture was placed in an ice bath for 30 min. 1200 µL of phosphate buffer (0.1 M) was added to the mixture. To this, 1250 µL of H_2O_2 (0.066 M) was added. The absorbance was read immediately at 240 nm up to 1 min. Catalase activity was determined using 43.6 M cm^{-1} as the molar extinction coefficient.[21]

Glutathione
Platelets (250 µL) were treated with 375 µL of 4% sulfosalicylic acid and vortexed. The mixture was spun at 2500×g for 15 min. The supernatant (40 µL) was treated with 280 µL of 10 mmol/L DTNB (Ellman's reagent), and the absorbance was measured at 412 nm.[22]

Total antioxidant capacity-cupric ion-reducing antioxidant capacity (TAC^{CUPRAC})
The platelet samples (5 µL) were treated with 195 µL of bathocuproine disulfonic acid disodium salt (BCS) (0.25 mmol/L), and the initial absorbance was measured at 490 nm. 50 µL of $CuSO_4$ solution (0.5 mmol/L) was added, and after 3 min incubation at room temperature (RT), 50 µL EDTA solution (0.01 M) was added. The final absorbance was measured at 490 nm. Total antioxidant capacity was calculated using uric acid as the standard.[23]

Oxidative stress markers
Superoxides
200 µL of cytochrome C (160 µmol/L) was added to 100 µL of platelet samples and the mixture was incubated for 10 min at 37°C. The absorbance was spectrophotometrically read at 550 nm to measure the reduction of cytochrome C.[24]

Nitrites
The platelet samples (100 µL) were mixed with 100 µL of Griess reagent. The mixture was incubated in the dark at RT. The absorbance was read at 548 nm. The amount of nitrites was determined using sodium nitrite as the standard.[25]

Thiobarbituric acid reactive substances (TBARS)
Platelets (100 µL) were placed in an ice bath for 10 min, and an equal volume of 20% (v/v) cold trichloroacetic acid (TCA) in 0.6 M HCl was added. The mixture was spun at 2000 rpm for 15 min and 40 µL of 0.12 M thiobarbituric acid (TBA) solution (in 0.26 M Tris at pH 7.0) was added to the supernatant. The mixture was boiled for 15 min and the samples were read at 532 nm.[26]

Protein sulfhydryls
Platelet samples (200 µL) were diluted with 1.5 mL of 0.08 M sodium phosphate buffer (0.5 mg/mL of Na_2-EDTA, and 2% sodium dodecyl sulfate). 0.1 mL of DTNB was added to the mixture and left for 15 min at RT. The absorbance was read at 412 nm.[27]

Advanced oxidation protein products (AOPP)
400 µL of samples were diluted with 1.2 mL of phosphate buffer and incubated with 200 µL potassium chloride (1.16 M). Absorbance was read at 340 nm immediately after adding 400 µL of acetic acid.[28]

Platelet functions and metabolism
Platelet aggregation
Platelet samples (100 µL) were incubated with collagen (100 µL, 2.0 µg/ml) for 10 min at 37°C in a V-bottom 96-well plate. The above procedure was repeated without collagen. The plates were subjected to shaking for 60 s, and the absorbance was recorded at 405 nm.[29]

ATP secretion
The platelet samples were incubated with 25 µL of collagen (2.0 µg/ml) for 10 min at 37°C. The mixture was treated with 1.2 M perchloric acid. The absorbance was measured at 260 nm. The amount of adenine nucleotides secreted was calculated using ATP as a standard.[30]

Glucose
Glucose was estimated using the Autospan Gold kit (Arkray, India), following the manufacturer's instructions based on the glucose oxidase-peroxidase (GOD-POD) method. The absorbance was read at 546 nm.[31]

pH
The pH of platelet samples was measured using the pH strips (Fisher Scientific).

Lactate dehydrogenase (LDH)
The platelet samples (10 µL) were treated with 1 ml of 4:1 mixture of reagent 1 (80 mmol/L Tris, 200 mmol/L NaOH, and 1.6 mM pyruvate) and reagent 2 (0.2 mmol/L nicotinamide adenine dinucleotide (NAD)) and incubated at 37°C for 3 min. The absorbance was measured at 340 nm for 3 min.[32]

Protein
500 µL of platelets were incubated with 2.5 mL alkaline $CuSO_4$ (50:1 mixture of 2% Na_2CO_3 and 0.5% $CuSO_4$) at RT for 15 min. 0.1 mL of Folin-phenol reagent (1:2 diluted in distilled water) was added to the above mixture. This was incubated at RT for 30 min. Absorbance was read at 660 nm. Bovine serum albumin (BSA) was used as the standard.[33]

Statistical analysis
Results are expressed as mean ±SE (n=5). One-way ANOVA was performed using GraphPad Prism 6 Soft-

ware (San Diego, CA, USA), followed by Bonferroni post-test. The values were considered significant at $p<0.05$.

Results

The variations occurring in FRI were compared against free radical-induced treated with melatonin antioxidant (FRI+AO) and controls. Variations occurring in melatonin antioxidant (AO) were compared against controls.

Antioxidant defenses

Superoxide dismutase activity decreased by 59% and 36% in FRI and FRI+AO, respectively, compared to the controls. SOD activity increased by 58% in FRI+AO against FRI (Table 1).

Catalase activity was 52% higher in AO compared to controls. It was 19% higher in FRI+AO compared to FRI (Table 1). Glutathione was 23% higher in AO than in controls (Table 1). TAC_{CUPRAC} was ~23% lower in FRI+AO than in controls and AO (Table 1).

Table 1. Antioxidant defenses in platelets exposed to AAPH-induced OS*

Groups	Superoxide dismutase (U/mg protein)	Catalase (U x 10^{-4}/mg protein)	Glutathione (mmol/mg protein)	Total antioxidant capacity (TAC_{CUPRAC}) (µmol uric acid equivalents/L)
Controls	5.11±0.5	10.00±2.3	3.64±0.5	840.2±123.7
FRI	2.06±0.2	7.65±2.0	3.37±0.3	766.93±64.6
AO	4.83±1.1	15.21 ±5.3	4.52±0.6	893.64±74.4
FRI + AO	3.26±0.8	9.14 ±2.3	3.56±0.2	658.00±56.5

* results are expressed as mean±SE (n=5), $p>0.05$

Oxidative stress

Superoxide levels were 41% and 53% higher than controls and AO, respectively. The levels were significantly higher by 75% ($p<0.05$) in FRI+AO compared to FRI (Fig. 1A).

Nitrites were 25% elevated in FRI+AO than in controls. However, it was maintained in all the other groups at similar levels (Fig. 1B).

TBARS increased in FRI by 12% and decreased in AO by 12% compared to Controls. It decreased by 24% in FRI+AO compared to FRI (Fig. 2A). AOPP was significantly higher (120%, $p<0.05$) in AO with respect to Controls. AOPP also increased by 66% in FRI+AO compared to FRI (Fig. 2B). P-SH levels were similar in all the experimental groups. However, it increased by 20% in AO compared to controls (Fig. 2C).

Platelet function and metabolism

Aggregation with collagen was significantly lower (~62%, $p<0.05$) in the FRI+AO compared to FRI and AO. Aggregation without collagen was similar in all other groups (Fig. 3B).

ATP secretion increased significantly in FRI+AO by 12% ($p<0.001$) compared to controls. It increased significantly by 17% ($p<0.0001$) and 8% ($p<0.01$) in FRI+AO compared to FRI and AO, respectively (Fig. 3B).

Glucose increased by ~20% in FRI and FRI+AO compared to controls (Fig. 4A). pH was similar in all the groups (Fig. 4B). LDH decreased by 17% in FRI compared to controls. There was an increment of 55% in FRI+AO compared to FRI (Fig. 4C).

Discussion

Free radicals play a crucial role in homeostasis and redox mechanisms in platelets.[4] AAPH generates peroxyl radicals that induce OS and is therefore employed to establish OS models. This study assessed the modulations in platelets by melatonin during AAPH-induced OS.

FRI vs. controls

Antioxidant enzymes act as the primary line of defense during OS and reflect the redox state of platelets. Peroxyl radicals can potentially inactivate the susceptible enzymatic active sites.[34] Therefore, superoxide dismutase (SOD) and catalase (CAT) lowered in FRI compared to controls. Notably, AAPH predominantly generates peroxyl radicals by thermal decomposition. These highly reactive radicals abstract hydrogen atoms from the lipids of the cell and its membrane to form lipid hydroperoxides (LOOH).[35] These lipid hydroperoxides are unstable and can decompose to form more reactive radicals, perpetuating the chain reaction of lipid peroxidation and leading to cell damage.[10] This corroborates with the higher TBARS levels, which also reflected in lowered TAC in FRI. Platelet aggregation increases during OS, activating a cascade of events leading to thrombus formation and posing a great risk in platelet disorders.[4] Aggregation in the presence of collagen increased in FRI, suggesting platelet stimulation due to redox changes. This can also be attributed to increased glucose metabolism and decreased LDH in FRI due to the aerobic state of platelets under in vitro conditions.

AO vs. controls

Melatonin, a powerful antioxidant, can penetrate the platelets due to its lipophilic nature. It stimulates endogenous antioxidant enzymes as seen in the increase in activity of SOD, CAT, and GSH in the AO. Melatonin directly scavenges hydroxyl radicals and indirectly detoxifies H_2O_2, superoxide, and peroxyl radicals through its metabolites.[12,36] This can be attributed to its powerful antioxidant capacity as it can bind to the Fe^{2+} ions and prevent the Fenton reaction. Therefore, melatonin lowers the susceptibility of membranes to oxidative damage and improves their stability.[37] Consequently, lipid peroxidation significantly lowered in melatonin-treated platelets compared to controls. Sulfhydryls are susceptible to oxidation and regulate the protein structure and function.[38] They are also

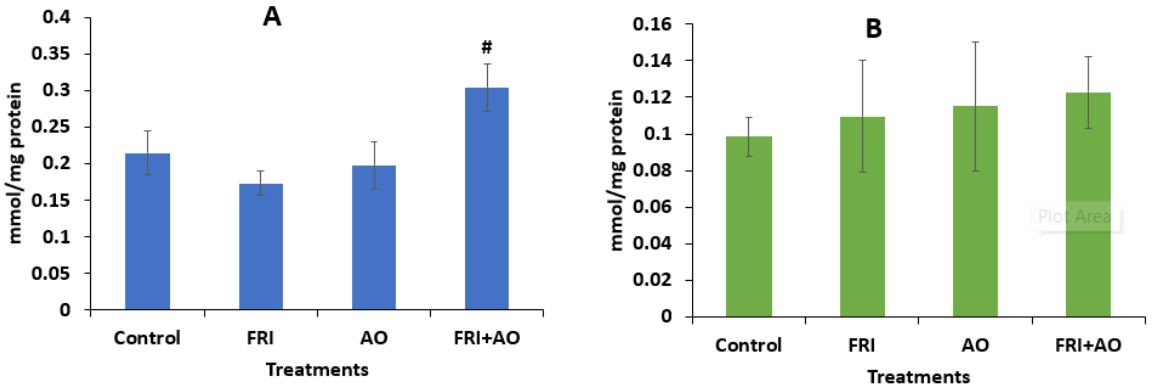

Fig. 1. A: Superoxides in platelets exposed to AAPH-induced OS expressed as mean±SE (n=5), B: Nitrites in platelets exposed to AAPH-induced OS represented as Mean±SE (n=5), # indicates significant difference with respect to FRI

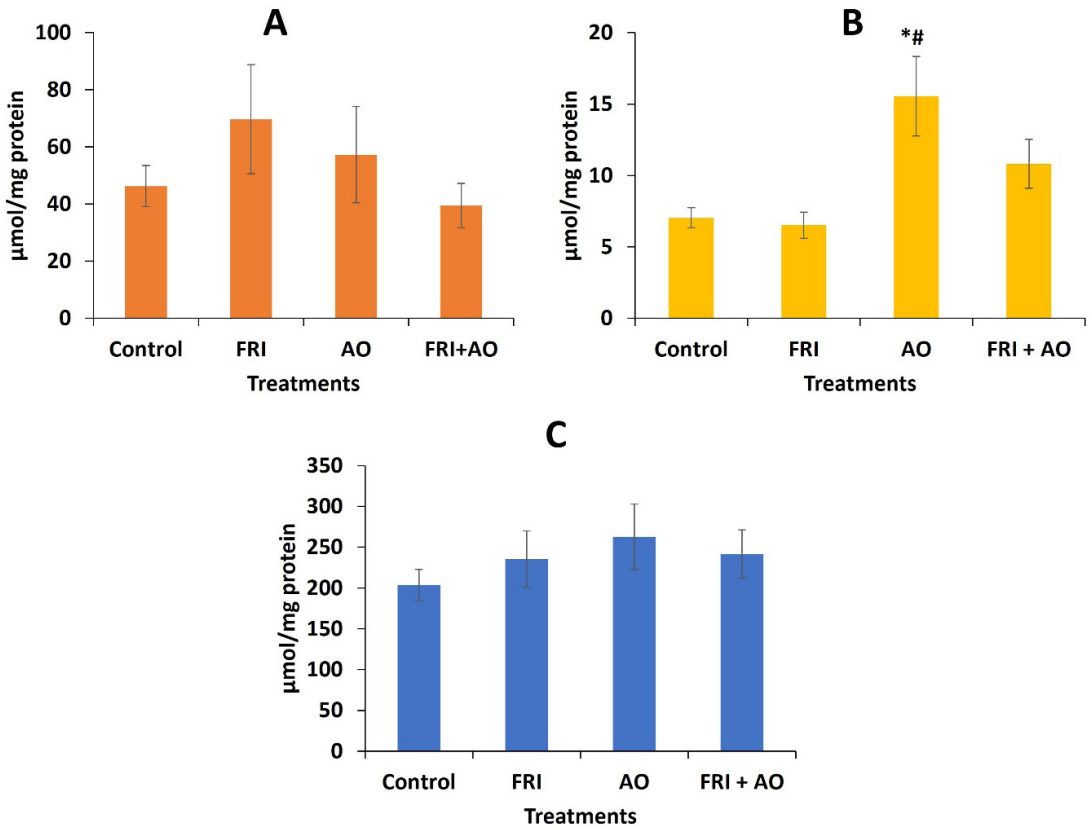

Fig. 2. A: TBARS in platelets exposed to AAPH-induced OS, B: AOPP in platelets exposed to AAPH-induced OS, C: Sulfhydryls in platelets exposed to AAPH-induced OS, results are expressed as mean±SE (n=5). * indicates significant difference with respect to controls, # indicates significant difference with respect to FRI

implicated in platelet activation and are required for adhesion via integrin αIIbβ3.[39] Protein sulfhydryls increased in AO, suggesting that melatonin was actively involved in protecting thiol groups by undergoing reversible oxidation. AOPP levels indicated that dityrosine linkages were formed, which can result in the cleavage of polypeptide chains and the formation of cross-linked protein aggregates.[40] AOPP levels increased in AO, suggesting that 1 mM melatonin could not prevent the formation of susceptible dityrosine linkages. Melatonin, as a hormone, can influence platelet functions in a concentration-dependent manner by altering its response to collagen and thrombin.[41] Melatonin increased the ATP secretion and aggregation (with collagen) in AO compared to the Controls, indicating platelet responsiveness to collagen and, clot formation. Melatonin regulates the pathways involved in platelet activation, thereby augmenting platelet responsiveness to agonists. Glucose and LDH levels were maintained in AO and controls, suggesting that melatonin did not intervene in platelet metabolism.

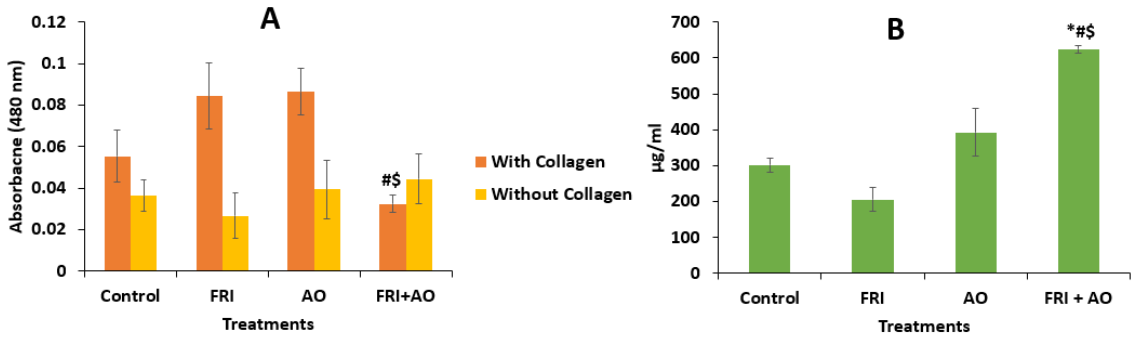

Fig. 3. A: Aggregation (with and without collagen) in platelets exposed to AAPH-induced OS, B: ATP secretion in platelets exposed to AAPH-induced OS, results are expressed as mean±SE (n=5), * indicates significant difference with respect to controls, # indicates significant difference with respect to FRI, $ indicates significant difference with respect to AO

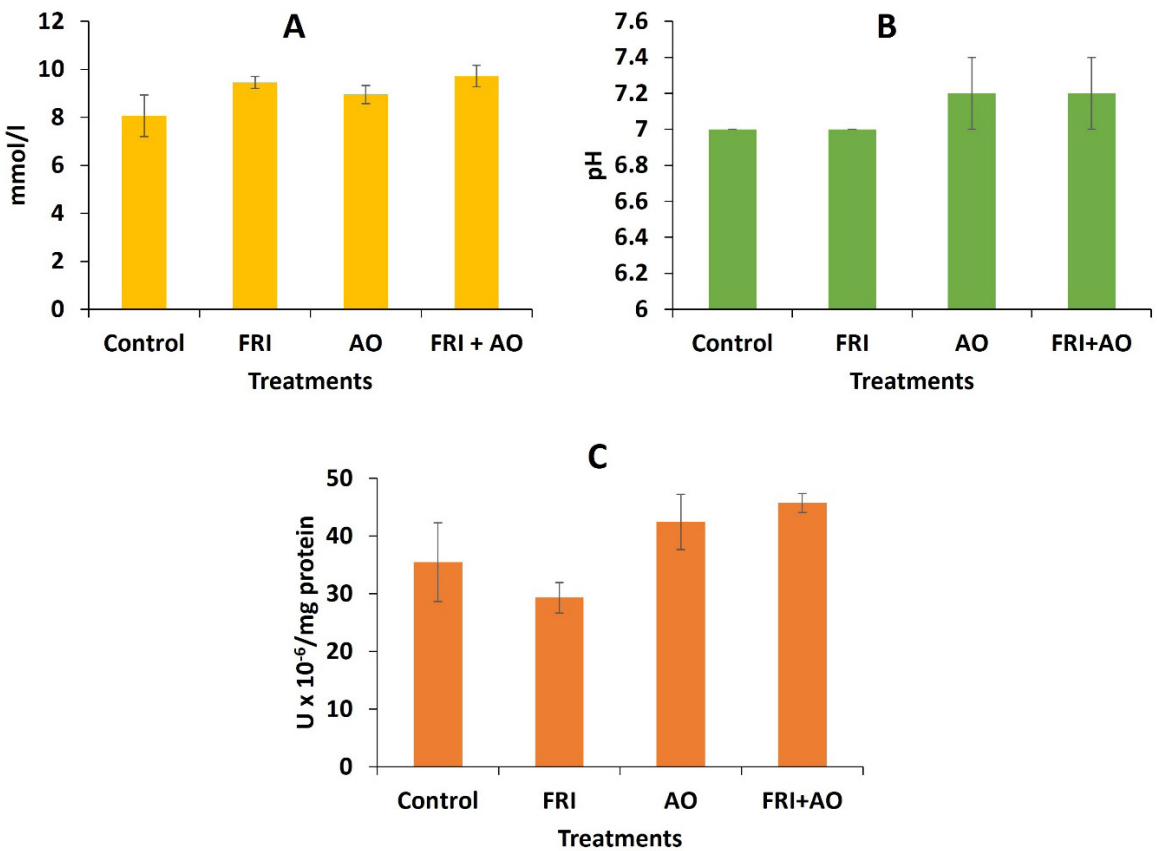

Fig. 4. A: Glucose in platelets exposed to AAPH-induced OS, B: pH in platelets exposed to AAPH-induced OS, C: LDH in platelets exposed to AAPH-induced OS, results are expressed as mean±SE (n=5), the changes between the groups were statistically insignificant

FRI+AO vs. FRI

The peroxyl radicals generated by AAPH are scavenged by melatonin using its two methoxy groups and an indole ring by a single-electron transfer (SET) mechanism.[34] Melatonin elevated the activity of SOD indirectly by increasing the production of superoxides in FRI+AO compared to FRI. CAT activity and GSH levels suggest that melatonin was involved in directly scavenging generated H_2O_2, as one molecule of melatonin is capable of scavenging two hydroxyl radicals. Melatonin has been protective against the catalase inactivation induced by AAPH, suggesting its involvement in reducing the oxidation of critical residues in these enzymes.[42] Nonetheless, elevated superoxide levels in FRI+AO signify the oxidative modulations due to AAPH in platelets, as superoxides are the primary and highly reactive species generated by physiological processes. Melatonin protects the lipids from oxidative damage by directly scavenging peroxyl radicals. It also actively protects the lipids due to their lipophilic nature, by altering the

membrane's physical properties at the peroxidation site.[12] TBARS decreased in FRI+AO, suggesting that lipid peroxidation was alleviated in platelets. This can be attributed to the SET mechanism, where peroxyl radicals are converted into stable molecules, thereby terminating the lipid peroxidation chain reaction.[34] Protein sulfhydryls were maintained in FRI+AO and FRI, suggesting that glutathione underwent reversible oxidation to protect SH groups to a certain extent.[4] However, ROS directly attacks the proteins by oxidizing certain amino acid residues, particularly those with sulfur-containing side chains, aromatic rings, or histidine residues.[1] AOPP increased in FRI+AO, suggesting that elevated superoxides might have rendered susceptible proteins to oxidation. Platelet aggregation in the presence of collagen significantly reduced in the FRI+AO owing to the increase in superoxide levels and LDH compared to FRI. ATP secretion significantly increased in the FRI+AO compared to FRI, indicating the increase in platelet sensitivity to collagen as a result of OS. This might be attributed to the biological defense mechanism of melatonin during OS by inhibiting prostaglandin synthesis, and can have implications in cardiovascular diseases.[43,44] Platelet metabolism was maintained as demonstrated by glucose levels. However, reactive species generated by AAPH affected platelet functions in FRI, and melatonin exposure benefited the platelets by modulating oxidative stress and platelet functions.

This study has potential clinical implications for platelet disorders. One of the major causes of platelet disorders is oxidative stress, as platelets actively generate ROS during activation. Antioxidants such as melatonin can modulate the physiological conditions and alleviate oxidative damage in platelets. The findings also indicate that melatonin can be explored in therapeutics for platelet disorders.

Study limitations

Melatonin is a hormone and is under constant regulation. A probable change in responses to physiological factors under in vivo conditions is possible.

Conclusion

Melatonin enhanced antioxidant defenses and attenuated lipid peroxidation in platelets at 1 mM concentration. It ameliorated the platelet function and maintained metabolism during AAPH-induced OS. These findings indicate that melatonin can modulate antioxidant capacity and alleviate oxidative stress in platelets. This suggests that melatonin plays a beneficial role in regulating redox mechanisms. Furthermore, in vitro platelet OS models provide insights into the implications of OS in platelet pathophysiology. Therefore, this study lays the foundation for further in vivo studies, which can contribute to therapeutic applications.

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Declarations

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Author contributions

Conceptualization, V.R.; Methodology, V.R.; Validation, V.R.; Formal Analysis, A.B.A. and M.C.R.; Investigation, A.B.A. and M.C.R.; Resources, V.R.; Data Curation, A.B.A.; Writing – Original Draft Preparation, A.B.A.; Writing – Review & Editing, V.R.; Visualization, V.R.; Supervision, V.R.; Project Administration, V.R.

Conflicts of interests

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethics approval

Animal care and maintenance were according to the guidelines of Institutional Ethical Committee (1810/PO/RcBizbt/S/15/CCSEA).

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