Review

Melatonin is a potential therapeutic molecule for oxidative stress induced red blood cell (RBC) injury: A review

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ABSTRACT

Red blood cells (RBCs) or erythrocytes are highly vulnerable to oxidative stress due to their absence of nuclei and mitochondria, presence of iron containing heme and high amounts of fatty acids in their uniquely constructed lipid bilayer membrane. The principal function of RBCs is to carry oxygen to tissues. Thus, RBCs have to pass through the micro-capillaries in which it requires these cells exhibits high structural deformability and great elasticity. The intact cytoskeletal architecture and proper membrane fluidity of RBCs are crucial for their deformability. Many factors can jeopardize the structural and functional harmony of RBCs. One of them is ROS which causes RBC oxidative injuries manifested by hemolytic anaemia such as occurring in β-thalassemia. Melatonin, as a potent free radical scavenger and antioxidant, can effectively protect against RBC oxidative injuries. In addition, melatonin chelates the free iron and upregulates gene expression of antioxidant enzymes of RBCs. Melatonin is synthesized and highly accumulated in RBCs to exhibit the on-site protection. All of these indicate that melatonin is a best molecule to preserve the structural and functional intactness of RBCs. This review tries to update the current development in the field and suggests the potential utility of melatonin on the RBC related disorders.

Key words: erythrocyte, red blood cell (RBC), oxidative stress, antioxidants, melatonin. β-thalassemia, anaemia.

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1. INTRODUCTION

Red blood cell (RBC) is a key component of life for vertebrates. Its primary functions are to carry and deliver oxygen to cells and in turn, to transport carbon dioxide out of the body. In this way, it helps to maintain the systemic acid-base equilibrium of organisms (1). Moreover, RBC serves as a redox state regulator by keeping the delicate balance between highly oxidative molecule (such as haemoglobin) and antioxidants (2).

Deformability is the primary criteria for RBC to pass through the microcirculation and deliver oxygen as well as nutrients to every part of the tissues and cells (3). For this purpose, the unique structure of RBC contributes to its proper functional state. RBC, except its ability of self-defence, can also protect other organs and tissues from deleterious oxidative stress due to its well-equipped antioxidant machineries (4, 5). It acts as the unique mobile free radical scavenger (6) and sensor of hypoxic condition occurring in the tissues. It releases nitric oxide (NO) as a signal to regulate the oxygen need in oxygen deprived tissues (7). Oxygen metabolism produces reactive oxygen species (ROS). Organisms have developed the endogenous antioxidant system during evolution to detoxify the harmful ROS. Imbalance of ROS production and detoxification leads to oxidative stress in organisms. In addition to the endogenous ROS formed as the by-products of aerobic respiration, several exogenous sources of ROS formation are also responsible for oxidative stress in organisms (8). Many clinical and patho-physiological conditions are attributed to the RBC oxidative stress (9). Lack of nuclei and mitochondria in RBC makes it incapable to synthesize new antioxidant enzymes and this increases the vulnerability of RBCs to oxidative stress (10, 11). Hemoglobin, the principle molecule being responsible for oxygen delivery, mostly remains in oxygenated status and subsequently leads to production of reactive oxygen intermediates. Additionally, the active heme component of hemoglobin contains iron in its ferrous state and when it binds to oxygen, the ferrous iron catalyses Fenton reaction, a prominent way of hydroxyl radical generation (12). It has been estimated that roughly 3% of hemoglobin undergoes autoxidation every 24 hours to produce methemoglobin (hemoglobin iron in ferric state) accompanied with formation of deleterious superoxide anion (13), which will be further amplified by many folds under stressful conditions (5). The released ferric iron from methemoglobin induces Haber-Weiss reaction to generate additional hydroxyl radicals (2). As mentioned above, many exogenous insults also cause RBC oxidative stress. Sometimes, these are the major causative factors related to RBC injuries (14). Oxidative stress of RBC has drawn a great attention of researchers since RBC can serve as a potent sensor of stress signals in mammals (15). Structurally, RBC has to experience tremendous osmotic stress when it passes through lung and kidney (16). The specialized membrane of RBC consists of large amounts of poly unsaturated fatty acids (PUFA) which are highly susceptible to peroxidation. RBC promotes the release of NO and generation of superoxide anion. When these two species interact each other they form highly reactive peroxynitrite. Peroxynitrite further damages the lipids and proteins and results in membrane structural disorientation and RBC dysfunction (17- 19). These features of RBC make it be a suitable model to study oxidative hemolytic and neurodegenerative diseases. These disease models also provide the opportunities for researchers to understand the potential mechanisms how oxidative stress progresses in spite of the presence of strong endogenous antioxidant pool in RBCs.

2. DIVERSE MODES OF OXIDATIVE STRESS IN RBC

RBCs are a suitable single cell model which is frequently used for studying ROS generation and hemolytic oxidative damage (20). Many naturally occurring substances, chemicals and medicines can cause RBC oxidative stress as discussed below (Figure 1):

2.1. Medicines and chemicals.

Atorvastatin, a medicine conventionally used to treat hypercholesterolemia, causes blebbing in RBC membrane and eryptosis due to oxidative stress (21). Hydrogen peroxide (H_2O_2) induces excess oxidative stress in erythrocytes by disturbing their deformability and elasticity (22-24). Band 3 proteins, the chloride-bicarbonate exchanger of RBC, responsible

for maintaining the homeostatic condition of erythrocyte is dismantled by H_2O_2 (25). Increased ROS and lipid peroxidation are observed in RBC of zebrafish when they are exposed to bisphenol A, a potential endocrine disruptor (26). Bexarotene is a potent drug used to treat malignancy. When human RBCs are treated with this medicine the ROS production and phosphatidylserine translocation are significantly increased (27). Another anti malignant medicine, phenoxodiol, also induces RBC membrane asymmetry by translocating phosphatidylserine in outer leaflet, a biomarker of apoptosis (28). Tamoxifen, an antiestrogenic medicine, used to treat breast cancer can induce hemolytic anaemia by destructing the skeletal protein structure of RBC membrane, principally by releasing peripheral and cytosolic proteins from Band 3 proteins (29). Opioids decrease RBC deformability as a result of occlusion of calcium (Ca^{2+}) pump, responsible for extrusion of intracellular Ca^{2+} and contributing in maintaining probity of RBC structure (30).

2.2. Heavy metals.

Heavy metals are toxic environmental insults to the RBCs. Heavy metals cause RBC toxicity manifested by altered morphology and increased stress markers (31). Highly toxic and pervasive heavy metal, mercury (Hg), induces oxidative stress in RBCs with a significant rise in ROS level and a concomitant decrease in the level of anti-oxidant glutathione (32). Hg also leads to hemoglobin oxidation and extensive inhibition of the enzymes involved in glutathione cycle, jeopardizing the free radical quenching ability of antioxidant machineries in RBC (33). NO synthesis is decreased in Hg exposed RBC due to the suppression of NO synthase (NOS) activity (34, 35). Reduced activities of potent antioxidant enzymes including catalase and glutathione peroxidase (GPx) are found in RBCs of eurasian eagle owls inhabited in the industrial area with cadmium (Cd), lead (Pb) and Hg pollution (36). Pb is known to alter the compositions of RBC membrane and to hamper hemoglobin synthesis (37-39). Activity of γ-aminolevulinate dehydratase, a major enzyme responsible for heme synthesis, was hindered in painters and battery workers exposed to lead and cadmium with the situation of redox imbalance (40). Hemoglobin and RBC content are significantly reduced in male wistar rats when cadmium chloride was given orally at a dose of 10mg/kg body weight (41). Similarly, oxidative stress occurs in male wistar rats when these animals received cadmium treatment (42). An increase in energy expense of RBC has been recorded in common carp exposed to Pb, Cd, copper (Cu) and zinc (Zn) (43). The adverse effects of waterborne metals on the oxidative biomarkers of RBC, especially on acetylcholine esterase, a crucial enzyme to keep RBC membrane properly functional, were observed in dice snakes from contaminated area of Serbia (44). RBCs are the major sites for lead's accumulation (45) and heavy metal exposure significantly suppresses the activities of catalase and GPx (46-48). When human RBCs were incubated with different doses of Cu, chromium (Cr), Pb and Zn their MDA levels were significantly enhanced (49). Long term of Cr exposure reduces activities of superoxide dismutase (SOD) and catalase, making erythrocytes more susceptible to oxidative stress (50). Collectively, RBCs are vulnerable to heavy metal toxicity which inhibits activities of biosynthetic enzymes and increases lipid peroxidation of RBC membrane, thus menacing RBC structure-function relationship (51- 53).

2.3. Cigarette smoking.

Abundance of active ingredients including aldehydes, heavy metals, nitrosamines, hydrogen cyanide, metallic ions, hydroxyl radicals and other oxidants in cigarette make smoking a harmful for human health, especially, it is toxic to RBC. Smoking causes membrane lipid peroxidation, desensitization of endogenous antioxidants and RBC

hemolysis (54- 56). Consumption of nicotine and cotinine, the two major constituents of cigarette, significantly reduces the level of sulfhydryl groups (-SH) in proteins residing in RBC membrane and these -SHs are essential for maintaining membrane stability by combating with ROS (57, 58). RBC suspension exposed to cigarette smoking leads to a large quantity of ROS formation (59). Increased systemic oxidative stress and altered RBC redox state are found in smokers (60). A strong correlation between smoking induced oxidative stress and the acute myocardial infarction has been identified (61). A significant increase in RBC count among smokers provides supportive information that smoking induces hypoxic state in human bodies (62). Consequently, the hemoglobin level in RBC is significantly increased both in male and female smokers (63, 64) compared to the non-smokers (65). RBC oxidative stress causes suicidal erythrocyte death i.e.; eryptosis which is highly correlated with the occurrence of chronic inflammatory diseases (66) and high level of superoxide anion in smokers (67). The RBC membrane enriched with polyunsaturated fatty acid is the target of oxidants and toxins of cigarette smoking and it is also the vulnerable place to generate large scale of ROS from smoking (54, 68, 69). RBC membrane injury leads to hemolysis and destruction of membrane asymmetry (58, 67). The smoking-induced consequential morphological changes in RBC were also visualized with the high resolution techniques (70, 71). Evidence showed that many chemicals generated by the smoking oxidatively attacked haemoglobin to form haemoglobin adduct which impeded normal oxygen carrying function of RBC (72).

2.4. High fat diet (HFD).

HFD is another factor to induce RBC pathology. For example, New Zealand white rabbit fed with HFD caused RBC hemolysis with increased methemoglobin and reduced oxyhemoglobin ratio (73). In hyperlipidemic rats feeding with 30% HFD for 8 weeks, the depletions of total thiol and glutathione levels in their RBCs result in the increased sensitivity of these RBCs to oxidative stress (74). The fragmentation and osmotic fragility of RBC are increased in HFD treated rats and the osmotic fragility is the principal marker of RBC membrane fluidity (75). A remarkable reduction of p55 and band 4.2 skeletal proteins in RBC membrane which are responsible for maintaining integrity of RBC structure was found in HFD treated rats accompanied with a collateral increase in malondialdehyde (MDA) level (76, 75). In mice, HFD also increases ROS production and phosphatidylserine externalization in RBC membrane which triggers proinflammatory response and macrophage activation to induce death signal in RBCs (77). Alterations in shape and deformability index of RBC were observed in the cholesterol fed rabbits (both *in vivo* and *in vitro*) with significant increase in membrane area (76, 78, 79), particularly in the peripheral side (80), leading to spicule formation (81). The elevated intracellular ROS production suppressed GSH level and decreased NO synthesis and these make RBCs be lack of response to hypoxic condition (82). The oxidative biomarkers including lipid peroxidation and protein carbonyl content in RBC increased in chronic alcohol consumers (equivalent to HFD) with increase in membrane cholesterol level. In addition, the density of membrane proteins such as band 3, 4.2, p58, demantin, actin, glycophorin significantly increased in alcoholics which may be an endeavour of RBC to respond to stressed situation (83, 84).

2.5. Xenobiotics.

Detoxification is a salient feature of RBC as they have the ability to remove toxins from the body (85). For this reason, it is important to examine the effects of several xenobiotics on morpho-functional status of RBC. Chlorfenvinphos, an organophosphate insecticide,

caused RBC hemolysis and methemoglobin formation by elevating the levels of TBARS and ROS (86). The general adverse effect of xenobiotics on RBC is to modify its morphologies which activate macrophages to eliminate the disformed RBC, resulting in deleterious anaemia (87). The underlying mechanism is related to oxidative stress induced by the xenobiotics. This was confirmed by the increased lipid peroxidation and decreased antioxidant enzyme activities in rat RBC exposed to a pesticide chlorpyrifos (88). The similar observations have been reported in different studies involved in chlorpyrifos and another contact-pesticide, endosulfan, on goat RBC (89). The elevated glutathione content and parallelly increased ROS production in RBCs treated with pyrethroid, a pesticide, reflects an early adaptive response of RBCs to oxidative stress (90). Due to the lipid peroxidation, the acetylcholine esterase activity of RBC membrane is also significantly reduced (90). The disturbed redox balance is the major cause of morpho-functional alterations in RBC afflicted by several organophosphate and carbamate pesticides (91- 95). The potassium leakage from RBC, related to the toxicity of organophosphates, is attributed to the increased membrane fragility of RBC (96). In addition, the impurities formed during pesticide synthesis also possess similar negative impacts on human RBC structure and function due to elevated ROS level (97). The strong correlation between usage of xenobiotics and RBC hemolysis has raised an alarming situation to consider minimizing the usage of harmful inorganic insecticides, fungicides and pesticides world widely (98- 101).

2.6. Training and exercise.

The excessive physical exercise also causes RBC hemolysis. A 40% shorter life span was reported in case of runners (102). Soccer players possess more oxidative stress in their RBCs compared to the RBCs of sedentary controlled subjects and the antioxidant supplementation is suggested to these players (103). Escalations of TBARS and methemoglobin have been found in exhaustive runners. The anionic transport and carbonic anhydrase activities of RBCs significantly decreased in those exhaustive-exercise athletes (104). Even the minimal exercises in case of untrained subjects can also lead to their RBC oxidative stress and modulation of RBC antioxidant system (105). Growing evidence has shown the adverse effects of exhaustive-exercise on the structures and functions of RBC (106). The exhaustive exercise breaks the normal glutathione balance cycle in RBCs along with diminished GSH level, giving a strong indication of RBC oxidative stress (107).

3. RBC DISORDERS AND OXIDATIVE STRESS

The associations of oxidative stress and RBC disorders are well documented. Thalassemia is an inherited autosomal disorder with defects in synthesis of either α globin chain (α-thalassemia) or β globin chain (β-thalassemia) (108, 109). This disorder is characterised by deficient erythropoiesis and short RBC life span due to the fact that diseased RBC membrane is less deformable and susceptible to lysis when passing through microcirculation (110- 112). All types of thalassemia exhibit clinical symptoms of anaemia, hepatosplenomegaly and RBC iron overload (109). The free iron is solely responsible for Fenton reaction which generates enormous amount of ROS to initiate membrane peroxidation and protein damage within RBCs (113, 114). Studies have uncovered the increased ROS generation and structural abnormalities in RBCs of thalassemia patients (110). Successful inhibition of iron overload-induced RBC oxidative stress by antioxidant treatment in thalassaemic patients further confirms that the redox imbalance is the major etiology of thalassemia (115, 116). The iron overload induced RBC oxidative stress can also be treated by the iron chelators that prevent subsequent Fenton reaction. (117). High levels of deleterious hydroxyl radical and low

amounts of antioxidants including GSH have been reported in thalassaemic patients as well as in the mouse thalassaemic model (118-121). As much as 90% of reduction in GSH/GSSG ratio (reduced glutathione: oxidised glutathione), is observed in thalassaemic patients compared to control subjects (122). Compromised levels of GSH and vitamin E and reduced activities of antioxidant enzymes including SOD and catalase have been reported in erythrocytes from α-thalassaemic patients (123). A negative correlation between GSH and MDA, GPx, glutathione reductase (GR) is a consequence of iron overload and oxidative stress in thalassaemic patients (124). The haemoglobin oxidation and superoxide anion formation in RBCs are the other characteristics of thalassemic symptoms (125). The attachments of three skeletal proteins, spectrin, actin and band 4.1 to RBC membrane are crucial for functional RBC. The impeded attachment in severe thalassemia causes hemolysis and consequently drives away RBCs from circulation (126). Thus, increasing the activity of antioxidant enzyme SOD in β-thalassemic children is a good strategy to combat this disorder (127). In addition to the endogenously occurring free irons in thalassaemic patients, the free irons derived from blood transfusion and oral iron therapy also trigger ROS generation (128). In summary, patients of α- or β-thalassemia have low RBC hemoglobin content due to alterations in the globin coding gene mutation. The binding capacity of these mutated hemoglobins to free irons is dramatically limited and the overloaded free irons in RBC trigger oxidative stress and RBC hemolysis which manifested as hemolytic anaemia clinically.

In contrast to iron overload anaemia, iron deficiency also causes hemolytic anaemia, characterised by decreased rate of hemoglobin synthesis and declined RBC formation. A major cause behind such anaemia is the prematurely removed RBCs from circulation (129, 130) due to the increased membrane hardness and decreased deformability of these RBCs (131-133). Iron deficiency prevailed within RBCs is associated with externalization of phosphatidylserine (PS) in outer leaflet of RBC membrane, a signature mark for RBC eryptosis (134, 135). This eryptosis is involved in the oxidative stress that triggers PS and calcium signalling in afflicted RBCs (135-137). The increased lipid peroxidation and decreased GPx activity further promotes the eryptosis (138- 143). Involvement of oxidative stress in iron deficiency anaemia is confirmed by use of antioxidant to correct this disorder (143, 144). The decreases in activities of potent antioxidant enzymes (SOD, catalase, GPx) significantly jeopardize the ability of RBCs to combat stressful situations (138, 141, 142, 145). In an iron-deficiency anaemia mice model, the RBC oxidative stress was manifested by decrease in methemoglobin and enhancement in fluorescent heme degradation product (146). Thus, both iron overload and deficiency caused ROS generation and hemolytic anaemia with different mechanisms. For former, the overload of free iron promotes the Fenton reaction and generates ROS. For the later, in case of iron deficients, due to unavailability of heme molecule, oxygen fails to bind with hemoglobin creating a hypoxic situation and this trigger increased oxygen partial pressure leading to enhancement in superoxide anion generation followed by hemoglobin autoxidation (147, 148). Hence, not only genetic alterations, but also oxidative stress is a key to deleterious situations associated with blood disorders (Figure 1).

4. RBC OXIDATIVE STRESS AND NEURODEGENERATIVE DISEASES

The neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), characterised by gradual loss of neurons, motor and cognitive functions, are associated with excessive ROS generation and oxidative stress (149, 150) in central nervous system (151, 152). Although the aggregation of misfolded proteins is the main etiology for these disorders, the abnormal RBCs also participate in its progression. For example, the altered RBC membrane proteins are found in AD patients (153). The band 3 protein in erythrocyte membrane undergoes breakdown at its transmembrane region in AD patients

(154). Exposure of RBC obtained from AD patients to oxidants triggers the binding of senescent antigen IgG, a relevant ageing marker of RBC membrane (155). Elevation of ion diffusion through the anion exchanger band 3 protein has been observed in persons with AD symptoms (156, 157), indicating functional anomalies of RBCs in AD patients. These morpho-functional alterations of RBC are always accompanied with neuronal cell death in AD patients (158), a positive correlation between RBC oxidative stress and progression of AD. Spin labelled RBC membrane from AD patient also exhibits the altered status of RBC in terms of its structure and function (159). When RBC membrane is exposed to different concentrations of Aβ1-42, the levels of TBARS increase in a concentration dependent manner (160). Aβ1-42 is responsible for forming senile plaques in progression of AD and it also causes RBC oxidative stress in response to amyloidosis (161). Other than brain, the presence of Aβ has been identified in RBC (162). Aβ interacts with RBC leading to its malfunction (163) due to Aβ mediated oxidative injury (164- 166). This oxidative injury enhances the occurrence of eryptosis (167), mainly triggered by caspase 3 mediated degradation of band 3 protein located in the RBC membrane (165). Malondialdehyde level is higher in RBCs of AD patients (168) and antioxidant treatment reduces this oxidative stress associated with the Aβ toxicity (169). RBC membrane of AD patients is highly fragile compared to that from the normal subjects (170). RBC from AD patients has the reduced SOD activity (171, 172) with a strong implication of oxidative stress occurred in RBCs of AD patients (173).

The elevated lipid peroxidation and reduced SOD activity are the characteristics of PD patient's RBCs (174, 175). A signature mark of PD patients is the accumulation of transferrin inside mitochondria and this leads to release of free iron (in ferrous state) potentiating Fenton reaction for hydroxyl radical generation (176, 177) and this gives a reason to use iron chelator as a medication to PD (177, 178). The presence of RBCs with eryptotic shape, loss of membrane phospholipid asymmetry altered RBC granularity and membrane elasticity are reported in PD patients, confirming the oxidative stress in RBC from PD patients (179).

5. DIABETES *MELLITUS* AND RBC OXIDATIVE STRESS – A CRUCIAL LINK

Oxidative stress plays a pivotal role in diabetic complications and RBC oxidative stress may attribute to the progression of diabetic conditions. Altered protein structure and redox system in the membrane of RBCs are found in type 2 diabetic patients (4, 180). Reductions in life span (181, 182), altered membrane phospholipid asymmetry (183), increased aggregation (184, 185) and multiple morpho-functional variations (186) are also the features of RBCs from the diabetic patients. Reduced ATP synthesis (187) in RBCs of diabetic patients due to oxidative stress causes glucose accumulation which is responsible for phosphatidylserine exposure. In addition to the functional anomalies mentioned above, structural variations of RBCs from diabetics are also visualized under the high-resolution imaging microscopy (188). Moreover, *in vivo* studies have shown that hyperglycaemia induces the over expression of IL6 which then, inhibits erythropoietin production (189, 190). The erythropoietin promotes generation of RBC and its inhibition finally results in anaemia (191). The increased oxidative stress in diabetic RBC further confirmed by the observations of either reduced antioxidant GSH or the activity of antioxidant enzyme, GPx (192).

6. GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH) - THE KEY STRESS RELIEVER IN RBCs

The metabolic enzyme, G6PDH, involved in hexose monophosphate (HMP) shunt pathway, participates in combating oxidative stress. This HMP shunt is the only source of

NADPH formation in RBCs since they lack citric acid cycle (193). RBCs require great amounts of GSH for their normal function under oxidative stress. GSH is synthesized by GR which requires NADPH as a cofactor. Hence, NADPH is a necessary factor to keep a balanced redox state within RBCs. RBC oxidative stress has been found in the case of G6PDH deficiency related to the X linked recessive disorder in which the NADPH is depleted and GSH regeneration is impeded (194, 195). Role of G6PDH in ameliorating the oxidative stress induced cell necrosis has been reported (196) and G6PDH knockout mice promotes oxidative stress (197).

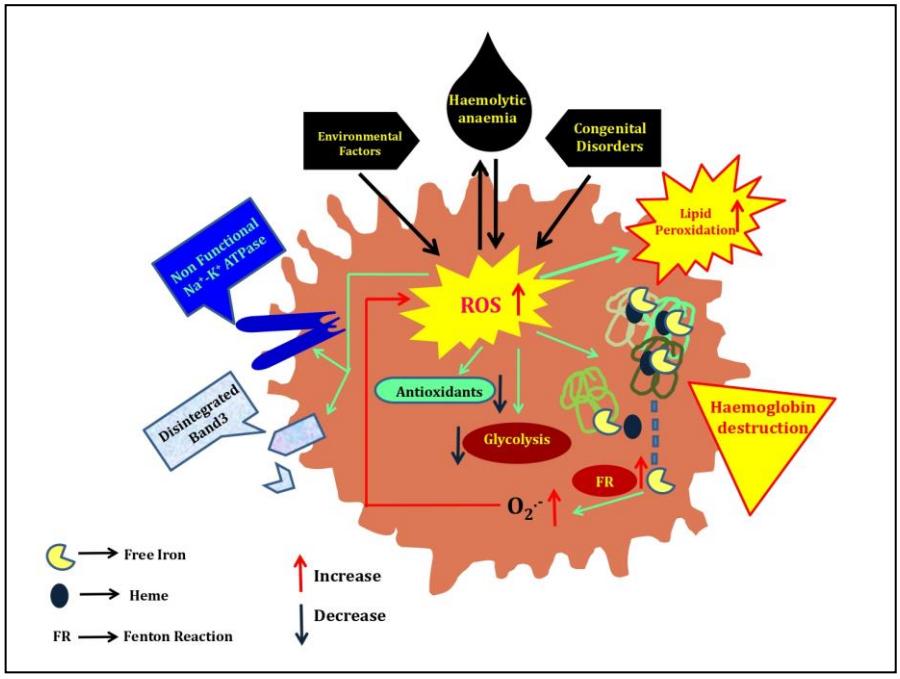

Figure 1: Different modes of oxidative stress induced harmful effects in RBC.

7. ANTIOXIDANTS- A PROTECTIVE MEASURE AGAINST OXIDATIVE STRESS OF RBCs

A wide range of molecules possess antioxidant capacity to protect cells as well as tissues from deleterious actions of ROS. These molecules are referred as antioxidants which act enzymatically or non-enzymatically to detoxify ROS. RBC is the most vulnerable cell affected by ROS and thus, it is shielded by diverse of endogenous and exogenous antioxidant machineries. For example, the exogenous antioxidants β-carotene and resveratrol can directly neutralize ROS in human erythrocytes (198) and they also stimulate the activities of the endogenous antioxidant enzymes, SOD and catalase, in RBCs (199, 200). The permeability of β-carotene through cell membrane (201) benefits its chelating to metals of diverse valency to reduce the cellular oxidative stress (202). The hepatic regenerator silymarin can reduce RBC oxidative stress induced by benzopyrene and H_2O_2 thus, preserves the functional status of RBCs (203). Natural antioxidants such as green tea leaf extract and ascorbic acid have the capacity to inhibit H_2O_2 and free iron induced membrane peroxidation which subsequently prevents hemolytic anaemia as well as the disruption of normal cellular function (204). Ascorbic acid administration also increases the tolerance of RBC of smokers to the oxidative stress (205). The phospholipid asymmetry, a signature mark of RBC apoptosis and the decreased RBC deformability are reversed by *Terminalia arjuna* bark extract via its antioxidant effect (206). The flavonoids including quercetin, rutin effectively ameliorate RBC hemolysis accompanied with a significant rise in sulfhydryl group containing molecules

(207). The oxidative damage during RBC storage is prevented by the commercial antioxidant mixtures (208) and the phenylhydrazine induced hemolytic anaemia and impaired erythropoiesis can be protected by the antioxidant vitamin C (209). The antioxidant cocktail is successfully used to treat β-thalassaemic patients in order to control the stress mediated hemoglobin destruction (210). Increased oxidative stress in diabetes due to overactive Mg^{2+} ATPase of RBC membrane causes elevation in glycolytic pathway and this reaction is inhibited by antioxidative effect of aqueous preparation of *Kalanchoe pinnata* leaves (211). Another antioxidant, N-acetylcysteine, can preserve the RBC cytoskeletal architecture and functional status in patients with non-insulin dependent diabetes *mellitus* (212). Thus, antioxidants play a critical role to protect RBCs from oxidative stress.

8. MELATONIN- A PHYLOGENETICLY ANCIENT ANTIOXIDANT

Melatonin, first isolated from bovine pineal gland as a neurohormone (213), has been later characterised as a potent free radical scavenger and antioxidant (214). This pineal indole has been reported to be phylogenetically ancient in evolution with its presence from bacteria (215) to human; therefore, it is classified as an oldest and versatile antioxidant (216). The ability of melatonin to forestall the oxidative stress and resultant molecular and/or cellular damage has been well documented (217). The potency of melatonin in scavenging hydroxyl (214, 218- 221), alkoxyl (222, 223) and peroxyl (224, 225) radicals is higher than those of other antioxidants. In addition, melatonin suppresses the generations of nitric oxide (226, 227) and singlet oxygen (228, 229) in neuronal and other tissues.

Mitochondria, the major sites of ROS generation, contain high level of melatonin compared to other compartments of cells (230- 233) and this is an on-site advantage of this molecule as an antioxidant superior to others. Actually, melatonin is synthesized, metabolized and functional in mitochondria and it is referred as the mitochondria targeted antioxidant (234). This indolamine exhibits proficiency to cross cell membrane and blood brain barrier (226, 235-237) to execute its protective effect. The metabolites of melatonin, produced from its reaction with free radicals, also possess potent antioxidant activity (238-242) along with a property to regenerate melatonin *per se* (243, 244). In addition, melatonin also has the capacity for restoration of the endogenous antioxidative system to broad its antioxidant spectrum. Even melatonin is reported to stimulate the activities of antioxidant enzymes (245), a breakthrough comes from the discoveries that melatonin upregulates mRNA expression of CuZnSOD and MnSOD which are responsible for dismutation of superoxide anion inside and outside the mitochondria (246). The upregulation of gene expression for GPx is also observed with melatonin treatment in neuroblastoma cells exposed to the Alzheimer amyloid peptide (247) giving an explicit view of its role in maintenance of cellular redox balance.

Its low urinary secretion (248) and high level of accumulation within cells by simple diffusion (249) and transportation are the major reasons to attribute high efficiency of melatonin as an antioxidant despite of its short half-life. Its none or low toxicity at vast range of doses for animals (250, 251) makes this molecule a widely acceptable antioxidant to detoxify the adverse effects of ROS. Moreover, the transition metal chelating capability of melatonin (252) increases its utilities as a protector of organisms under adverse stress situations.

9. MELATONIN IN SEVERAL LIFE-THREATENING DISORDERS

An excessive ROS generated in cells under stressful condition can be amended by melatonin exploiting the mechanisms described above. Notably, melatonin in circulation is responsible for its receptor mediated action. These receptors are present either in cell

membranes or in the intracellular organelles (253, 254). The direct free radical scavenging and indirect increase in other endogenous antioxidant level by acting on the receptors (255, 256) are the major antioxidant mechanisms of melatonin. Melatonin synthetic genes which are expressed in several important tissues and organs facilitate its on-site protective effects. Hence, this tiny indole can be shining armour against destructive free radicals.

Melatonin is a potent protector of cardiac damage induced by oxidative stress. The clinical trials have shown the protective effects of melatonin on cardiac arrhythmia (257), myocardial ischemia/reperfusion injury (258, 259- 261), its secondarily occurred liver and gastric tissue lesions (262, 263) and cardiac apoptosis related to the metabolic disturbance (264). The oxidative cardiac damage induced by high fat diet in rat is also protected by melatonin (265). The decrease in circulating level of melatonin shows high correlation with the occurrence of diabetes (266) and, thus, melatonin is used to enhance the bioavailability and functionality of metformin to treat type II diabetes *mellitus* (267). Based on the evidence, melatonin is suggested to be a promising molecule against stress induced diabetic cardiomyopathy (268). Furthermore, melatonin is also a potential remedy to inhibit non-steroidal anti-inflammatory drug (NSAID) induced gastric injury (269, 270) and the low endogenous melatonin synthesis has been associated with gastro-duodenal tissues and subsequent gastric injury (271, 272). Recently, heavy metal induced splenic injury was also reported to be mitigated by melatonin (273). Several studies give an optimistic view to prescribe melatonin as a curative medicine against neurodegenerative disorders and β-amyloid accumulation induced lipid peroxidation (274-276), mitochondrial dysfunction along with DNA lesion (277) in AD patients. Hence, melatonin is well accepted as a remedy to delay oxidative stress in ageing process which is distinctly elucidated in a study of life-long melatonin deficient rats (278).

10. MELATONIN PROTECTION AGAINST RBC INJURY INDUCED BY OXIDATIVE STRESS

RBCs are able to extract melatonin from circulation and its accumulation promotes the activity of GPx (279). RBCs also synthesize melatonin *de novo* (280) and this probably the only cell synthesizes melatonin without mitochondria. This ability further enhances the melatonin concentration in RBCs. Melatonin is considered as a redeemer to preserve structurally and functionally compromised RBCs from oxidative stress. For example, the cumene hydroperoxide treatment caused RBC hemolysis with increased osmotic fragility, membrane haemin and other degradation products formation while these alterations are reversed by melatonin application (281). In addition, the cumene hydroperoxide causes perferryl haemoglobin formation. This perferryl haemoglobin contains highly oxidant iron oxoferryl heme group which leads to methemoglobin formation and this reaction is blocked by melatonin treatment in the afflicted RBCs (282). The malondialdehyde, released into blood stream as an indicator of oxidative stress, acts as a stressor to RBC triggering its lysis and subsequently its destruction and these can also be cushioned with melatonin intervention (283). The mechanisms involved in protective measures are not only antioxidant activity but also the chelating property of melatonin. Melatonin chelates free iron, formed during RBC apoptosis and cause forceful destruction under stressed conditions, to inhibit Fenton reaction and subsequent ROS generation. Additionally, a favourable binding pattern of melatonin with the highly oxidant molecule phenylhydrazine has been reported in the *in vitro* study. By binding to phenylhydrazine, melatonin inhibits the ability of this molecule to generate ROS in RBC, a novel mechanism of melatonin's preventive role for RBC against excess ROS formation (284). Clinically, melatonin has been applied to athletes in maintaining their antioxidant level during preparatory period of competitions (285) with increased activities of catalase and GPx in their RBCs (286). Melatonin is also applied in RBC storage to eliminate

the detrimental effects of oxidative damages (287). The protective effect of melatonin on RBC oxidative stress has been observed in senescence accelerated mice (288). Both fluidity and mechanical stability of RBC are the central components to maintain its functionality. Cell membrane is a major compartment of subcellular melatonin distribution and its level is even greater than that in nucleus and cytosol (289). The small molecule size and the amphipathic characteristics make melatonin able to penetrate through every subcellular compartment to produce its antioxidative activity (290). It is suggested that its presence in the hydrophobic core of membrane can successfully scavenge nitroxide which is even present within groove of the bilayer (291).

On the other hand, studies indicate the possibility that melatonin preferentially localizes in heads of phospholipids where there is surplus of ROS within membrane (292). Melatonin enhances lipid dynamics by increasing the free motion of lipid molecules in cell membrane (293).

The effects of melatonin on glucose metabolism in RBC have been a focus and melatonin limits glucose uptake in tumour cells. The expressions of GLUT1 and GLUT4 in RBCs and the competition between melatonin and glucose to bind to these glucose transporters indicate that melatonin can be transported into inside of the cells via these glucose transporters (249). Melatonin protects hemolytic anaemia induced by oxidative stress and thus, enhances the levels of hemoglobin and RBC life span within circulation (209). The hemolytic anaemia as a result of G6PDH deficiency can be palliated by melatonin application (294). The hydrogen peroxide treatment disturbs the anion exchanger Band 3 protein, a crucial for RBC function; however, melatonin application reverses this in human RBC in an *in vitro* study (295). The probable protective mechanisms of melatonin on RBC oxidative injuries are illustrated in Figure 2.

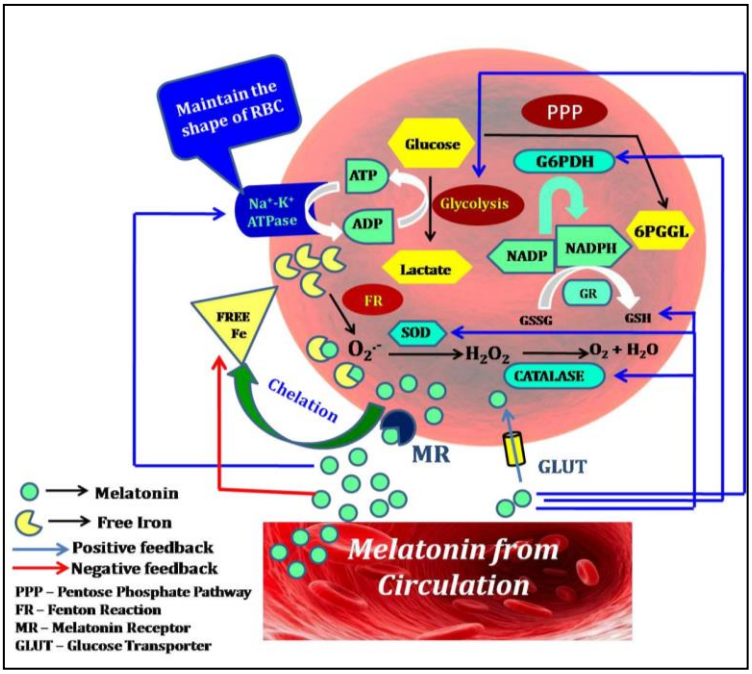

Figure 2: Different pathways underlying the protective actions of melatonin in combating oxidative stress in RBC.

11. CONCLUSION AND FUTURE PERSPECTIVE

An excessive ROS generation is inevitable for organisms under the oxidative environment of earth. Since RBC is the major oxygen transporter of mammals, this makes RBC more

vulnerable to oxidative stress. This review focuses on the influence of several environmental factors and congenital disorders on the morpho-functional integrity of RBC. It appears that endogenous antioxidants play critical role to preserve the functions of RBC under the oxidative stress. Among these antioxidants, melatonin is a choice by its unique feathers. RBCs can extract melatonin from circulation and they can also synthesize melatonin, therefore, high level of melatonin can be accumulated inside of RBCs to exert their on-site protective action. Melatonin directly scavenges the ROS, indirectly stimulates the activities of antioxidant enzymes and also chelates the free irons. All of these contribute to melatonin's potent protective effects on RBC oxidative damage. Hence, this review suggests melatonin as a restorative agent to overcome RBC injury caused by a variety of insults. It is our suggestion to use melatonin as a therapeutic agent to prevent RBC from hemolytic disorders.

AUTHOR'S CONTRIBUTION

Dr. DB and Dr. AC contributed to conception, revised the manuscript critically and approved it. AB prepared, drafted and edited the manuscript and figures. Dr. PKP contributed in editing the manuscript and figures.

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CONFLICT OF INTEREST.

Authors declare no conflict of interest.

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