Review

The potential of melatonin in the prevention and attenuation of oxidative hemolysis and myocardial injury from cd147 SARS-CoV-2 spike protein receptor binding

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ABSTRACT

The rapid escalation of pandemic health threats associated with the novel, pathogenic SARS-CoV-2 coronavirus poses unprecedented challenges as proven effective vaccines and drugs have yet to be produced. Refractory hypoxemia and myocardial injury have been observed as two of the major causes of fatality in COVID-19 patients. SARS-CoV-2 spike (S) protein binding to broadly expressed CD147 receptors on erythrocytes causes oxidative hemolysis that may result in refractory hypoxemia and myocardial injury. Both of these life-threatening conditions are further exacerbated by imbalance in ACE2 from spike (S) protein receptor binding. Dysregulation in the CD147-cyclophilin A signaling pathway, together with altered calcium signaling from SARS-CoV-2 ion channel activities, may contribute to hypercoagulation, thrombosis, and cardiac remodeling resulting in heart failure. Melatonin is an ancient pleiotropic molecule with recognized antioxidant properties that is essential for the protection of erythrocytes from oxidative hemolysis. Found in erythrocytes, melatonin can reverse hemolytic anemia, normalize heme synthesis, restore lymphocytes and platelet counts, and reduce vessel permeability during an acute hemolytic crisis by maintaining intracellular calcium homeostasis and reduction of oxidative stress. In acute hypoxic conditions, melatonin is cardioprotective via blunting of cardiopulmonary response to hypoxia and suppressing hypoxia pathways. Melatonin normalizes endothelial-dependent nitric oxide production to prevent multiple organ damage from hypercoagulability, thrombosis, and hypertension associated with oxidative hemolysis and ACE2 deficiency, protecting cardiomyocytes from hypertrophy. This review discusses the full potential of melatonin as a safe and effective therapeutic intervention for the prevention and attenuation of hemoglobinopathies, refractory hypoxemia and myocardial injury during critical COVID-19 infections.

Key words: melatonin, COVID-19, CD147, ACE2, hemolysis, hypoxia, myocardial injury, erythrocytes, platelets.

1. INTRODUCTION

The receptor-binding domain (RBD) of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein exhibits high affinity to angiotensin-converting enzyme 2 (ACE2) (1, 2). ACE2 is a homologue of ACE that degrades angiotensin I into angiotensin 1-9 and converts angiotensin II into angiotensin 1-7. Host cell entry and membrane fusion begins with the successful binding of spike protein RBD to ACE2 receptors using ACE2 molecules captured by RBDs (3). Furin enzyme cleavage at S1/S2 site may precede the priming of spike protein by host serine protease TMPRSS2 (4). ACE2 is highly expressed in cardiomyocytes, cardiac fibroblasts and coronary endothelial cells. An imbalance in ACE2 can result in blood pressure dysregulation and acceleration of heart failure progression. The possible downregulation of ACE2 protein expression as a result of SARS-CoV infection (5) may contribute to acute myocardial injury and cardiovascular system damage observed in COVID-19 patients (6-9). The negative correlation between ACE2 expression and COVID-19 fatality observed in an analysis of ACE2 expression quantitative loci (eQTLs) from GTEx in 30 tissues from thousands of individuals (10) further emphasizes the important essential physiological functions of ACE2 that could protect COVID-19 patients during infections.

The abundant expression of ACE2 receptors in differentiated airway epithelia (11) supports the initial observation of prevalent pneumonia that could evolve rapidly into acute respiratory distress syndrome (ARDS) in COVID-19 infections (11, 12,). Recent accounts from northern Italy describing COVID-19 patients who fulfilled the Berlin criteria of ARDS revealed atypical presentation of the ARDS syndrome. The severity of hypoxemia in these patients was disproportionate to their relatively normal lung capacities. The unexpected discrepancy between relatively high respiratory compliance of 50.2 ± 14.3 ml/cmH2O indicating acceptable lung gas volume and shunt fraction of 0.50 ± 0.11 has never been reported before in ARDS patients (13). In severe ARDS, the level of hypoxemia would be linearly associated with low respiratory system compliance (14).

While atypical ARDS features are being reported in COVID-19 patients in different countries (15), two cohort studies representing early data from hospitalized COVID-19 patients in Wuhan, China, identified the association between myocardial injury and high mortality rates (51.2%, 42/82) (16). Myocardial injury resulting in cardiac dysfunction and arrhythmia was found in 27.8% of COVID-19 patients in a case series study involving 187 patients (17). Both cohort studies found elevated troponin T (TnT) and troponin I (TnI) correlated with high mortality rates (59.6%, 31) compared to patients with normal troponin levels (16, 17). Mortality rate was unusually high (37.5%, 6/16) in COVID-19 patients without any prior history of cardiac disease (17).

The binding of ACE2 to SARS-CoV-2 spike protein RBD may explain the development of ARDS and myocardial injury in infected patients. The unexpected dissociation in severe hypoxemia and relatively normal lung capacity in critical COVID-19 patients remained largely unelucidated. The report by Liu *et al*. (18) of an in silico demonstration of the coordinated attacks by SARS-CoV-2 ORF1ab, ORF10, and ORF3a proteins on the 1-beta chain of heme in hemoglobin and the subsequent dissociation of iron ion, docking of viral protein ORF8 in porphyrin, presented the first working hypothesis that supports hemolysis in COVID-19 infection. The subsequent *in vitro* identification of CD147 as a novel receptor of SARS-CoV-2 spike protein (SP) by Wang *et al*. employing immuno-electron microscope to determine the localization and CD147-SP interaction demonstrated viral invasion of Vero E6 cells was facilitated by CD147/SP interactions (19). The development of refractory hypoxemia and myocardial injury can be the

direct result of CD147-SP interactions as CD147 are highly expressed in erythrocytes (20) and cardiomyocytes (21). The invasion of host cells by SARS-CoV-2 through CD147 as a novel receptor of SP could result in myocardial injury and extensive pathologies associated with oxidative hemolysis. Even though Meplazumab (anti-CD147 antibody) has been found to inhibit SARS-CoV-2 invasion by blocking CD147 (22), its use should be carefully considered.

2. DIRECT EFFECTS OF CD147/SPIKE PROTEIN INTERACTIONS

CD147, also known as Basigin or EMMPRIM, is a transmembrane glycoprotein with diverse pleiotropic functions, regulating both physiological processes in vision, spermatogenesis, and pathological processes in cancer involving apoptosis, cell proliferation and migration (23). The pleiotropic effects of CD147 is uniquely demonstrated in its ability to promote either lysis or fibrosis dependent on cell microenvironment and cell-cell interactions. Inflammation induces glycosylation of CD147, promoting adhesion, migration and enhancement of inflammatory signaling (NF-κB) and synthesis of matrix metalloproteinases (MMPs) (24, 25). CD147 has been deeply implicated in thrombotic and inflammatory pathways in coronary artery disease (CAD) progression due to its wide expression on many cell types including hematopoietic, endothelial cells, leukocytes, platelets, to name a few (26). In erythrocytes, CD147 promotes P. falciparum malaria parasite invasion of red blood cells serving as direct receptor for the parasite ligand PfRh5, in a way not dissimilar to the binding to spike protein of SARS-CoV-2 (27).

2.1. Mature erythrocyte plasma circulation and leukocytosis.

CD147 is an adhesion molecule that is expressed in all stages during the differentiation and maturation of erythrocytes. CD147 is essential for the expression of monocarboxylate transporter 1 (MCT1) on erythrocytes, forming a complex with MCT1 (28, 29). MCT1 facilitates the transport of lactate and pyruvate (30). Dysregulation in CD147/MCT1 can lead to the accumulation of lactate in erythrocytes, resulting in the disturbance of the lactate/pyruvate ratio required for normal glycolytic flow in mature erythrocytes (31). Blocking CD147 adhesion on plasma surfaces of mature erythrocytes completely disrupts their circulation, resulting in anemia as erythrocytes become trapped in the spleen, resulting in splenomegaly (32). In the inflammatory microenvironment of splenomegaly, CD147 can induce liver injury and fibrosis (33), as inflammation induces glycosylation of CD147 that can promote adhesion and migration (24). During inflammation, the spleen recruits leukocytes directly from the blood (34). The development of leukocytosis (14.1 x 103/uL) in COVID-19 infection has been reported (35). Leukocytosis in either normal or leukemic cells is often correlated with leukocyte adhesiveness/aggregation in the peripheral blood (36). During inflammation, leukocytes upregulate CD147, exacerbating cell-cell aggregation to cause significant fibrosis and liver injury. Blocking of CD147 in vivo can reduce leukocyte aggregation and significantly attenuate liver injury and fibrosis (37). In severe COVID-19 patients, liver dysfunctions marked by abnormal elevation in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) have also been observed (38). In view of the possible diverse effects of CD147 spike protein binding on red and white blood cells, further research and clarification of the exact role and function is urgently needed.

2.2. Lymphopenia.

Lymphopenia (lymphocytopenia) in COVID-19 patients can accurately predict disease severity and mortality (39). COVID-19 non-survivors have significantly lower lymphocyte counts than survivors, as functional exhaustion of lymphocytes is correlated to disease progression (40, 41). Critical COVID-19 patients were found with low lymphocyte counts (0.8 vs 1.0 $\times 10^9$; $P < 0.001$), higher neutrophil-to-lymphocyte ratio (5.5 vs 3.2; $P < 0.001$) that reflected high neutrophil counts (4.3 vs 3.2 $\times 10^9$; $P < 0.001$) (42). Lymphopenia in severe COVID-19 patients may be due to the active destruction of lymphocytes. Studies have demonstrated that SARS-CoV-2 could invade T-lymphocytes through spike protein receptor binding to CD147 (22, 43). The destruction of lymphocytes by CD147 SP invasion is extremely plausible since ACE2 is entirely absent in lymphocytes, while CD147 is highly expressed on activated T-lymphocytes (44). Lymphopenia is often accompanied by coagulopathy in COVID-19 patients (39). A high platelet to lymphocyte ratio has been correlated to COVID-19 disease severity, where patients with distinctly elevated platelet counts had longer average hospitalization (45).

2.3. Thrombocytopenia, coagulopathy and myocardial injury.

Hypercoagulability leading to widespread microvascular thrombosis, characterized by the depletion of platelets and coagulation proteins, results in life-threatening bleeding in coagulopathy (46). A meta-analysis of four studies involving 1,427 COVID-19 patients found thrombocytopenia (OR, 5.1; 95% CI, 1.8-14.6) to be correlated with a five-fold increase in risk of disease severity. A subgroup analysis detected mortality to be associated with an even lower platelet count (WMD, -48×10^9/L; 95% CI, -57 to -39×10^9/L) (47). A separate large-scale study involving 1,476 COVID-19 patients revealed that thrombocytopenia is strongly correlated to in-hospital mortality rate. A 92.1% mortality rate was associated with the group with the lowest platelet count (0, 50 x 10^9/L), and only 4.7% with the group with normal platelet count (150- x 10^9/L). Patients with platelet counts below 50 x 10^9/L had the highest mortality relative risk of 13.68 (95% CI 9.89-18.92) compared to 3.42 (95% confidence interval [CI] 2.36-4.96) for those with platelet counts between 100-150 x 10^9/L (48). Von Willebrand factor (VWF) is a platelet-adhesive protein produced by endothelial cells and megakaryocytes (49). The coagulation protein factor VIII must be complexed with VWF to avoid proteolysis and clearance (50). VWF is often found to be elevated, together with factor VIII in severe COVID-19 patients (51, 52). ADAMTS13 is a metalloprotease that cleaves von Willebrand factor to prevent thrombocytopenia. ADAMTS13 is downstream of CD147 (53, 54). CD147 is a novel platelet surface receptor that activates platelets (55). In humans, 20.45% +/- 1.63% of circulating platelets express CD147 (56). Binding of SP to CD147 can deactivate CD147, inhibiting ADAMTS13, while at the same time, CD147 SP receptor binding on platelets may induce platelet degranulation, leading to dysregulation in coagulation and possible destruction of platelets (57). CD147-Cyclophilin A (CypA) signaling can activate platelets, causing increased adhesion and thrombus formation *in vitro* and *in vivo* (57). 90% of COVID-19 inpatients with pneumonia with elevated D-dimer showed increased coagulation activity. A D-dimer value greater than 1 μg/mL (18·42, 2·64–128·55; p=0·0033) was associated with high mortality. 81% of non-survivors (44/54) had D-dimer values over 1 μg/mL, compared to 24% (28/118) in survivors (58). Hypercoagulation in COVID-19 patients (59) can be the result of hemolysis which has been shown to activate platelets *in vitro* and *in vivo* (60).

Myocardial ischemia/reperfusion injury is exacerbated by upregulation of platelet surface receptors such as CD147 that results in cardiac damage via activation of immune cells or modification of cardiovascular endothelium (61, 62). CD147 has recently been demonstrated to promote cardiac fibroblast proliferation. Elevated serum levels of CD147 in patients with heart failure predicted poor prognosis as CD147 is essential in the pathogenesis of cardiac hypertrophy, fibrosis and failure in humans and mice (63). In COVID-19 patients, 20% of non-survivors (11/54) were found to have platelet counts of $<100 \times 10^9$ per L, compared to only 1% (2/137) in survivors (58), while elevated platelet counts also indicated poor prognosis for COVID-19 patients (64). In a retrospective single-center study, 11.8% (32 of 271) of enrolled COVID-19 patients, 71/9% of whom were elderly with low lymphocyte count on admission, developed delayed-phase thrombocytopenia 3 to 4 weeks after symptom onset. These patients exhibited mean nadir platelet count at 86.0×10^9 /L (SD = 37.48) (65).

3. CD147/SP INTERACTIONS INDUCE ACUTE OXIDATIVE HEMOLYSIS

Erythrocytes are now understood to affect immune function with no less than 46 pro- and anti-inflammatory cytokines detected in red blood cell lysates of healthy individuals (66). The cytokine interleukin 1 beta (IL-1β) and its receptor IL1R1 have been demonstrated to enhance megakaryocyte maturation and activate platelets under proinflammatory, prothrombotic conditions in humans and mice (67). Extracellular hemoglobin (ECHb), when oxidized, is able to upregulate expression of NLRP3 inflammasome, activating IL-1β production in human umbilical vein endothelial cells (HUVECs) (68, 69). *In vivo*, hemolysis and heme have been shown to activate NLRP3 inflammasome in macrophages (70, 71). Elevated platelet counts in COVID-19 patients have been associated with increased cytokines that stimulate the release of platelets (72). Elevated levels of fibrinogen and fibrin degradation product D-dimer, as well as VWF and factor VIII, may indicate a dysregulation in the balance between coagulation and fibrinolysis (73). In critically ill COVID-19 patients, shutdown of fibrinolysis is significantly correlated with thrombotic complications (74). Patients with higher levels of hemolysis have increased buildup of platelet clots as extracellular Hb can increase platelet adhesion and thrombosis by interacting with the A1 domain of VWF (75). Injury to lung tissue and pulmonary endothelial cells can cause the activation, aggregation and retention of platelets in the lung (76). The lung is a major site where mature megakaryocytes release platelets. Approximately 50% of total platelets produced in the human body, or about 10 million platelets per hour, are generated in the lung (77). Lung injury or damage to pulmonary capillary beds can block megakaryocyte ruptures (78), resulting in reduction of platelet release and platelet synthesis (79). During acute lung injury or ventilator-induced lung injury, CD147 expression in lung endothelial and epithelial cells may be significantly upregulated (80, 81).

The invasion of host cells including lymphocytes by SARS-CoV-2 spike protein receptor binding to CD147 receptors has been clearly identified (19, 22, 43). The broad expression of CD147 on erythrocytes and platelets imply that SARS-CoV-2 can readily bind and fuse with erythrocytes and platelets. It is likely that spike protein fusion peptides and internal fusion peptides participate in cell entry by coronaviruses (82), causing hemolysis. Hemolysis describes the pathological condition when disruption or damage of erythrocyte plasma membrane results in the release of hemoglobin or other intracellular components to surrounding plasma. More than 30 grams of cell-free heme may be released from erythrocytes during hemolysis (83).

Erythrocytes are dependent upon a robust antioxidant system to maintain hemoglobin (Hb) in a reduced state that binds and transports oxygen and carbon dioxide throughout the body (84). Iron ions in the center of heme groups within hemoglobin can only bind oxygen in the reduced ferrous (Fe^{2+}) state (85). During hemolysis when the cell membranes of erythrocytes are disrupted or damaged, such as during an attack by SARS-CoV-2 spike protein receptor binding with CD147, Hb is rapidly released from ruptured erythrocytes, forming cell-free hemoglobin. Without adequate antioxidants in plasma, Hb becomes destabilized and participates in oxidative reactions resulting in formation of methemoglobin in the ferric ($Fe3^{+}$) state which can release free heme at much higher rates (86, 87). Under oxidative conditions with un-neutralized H_2O_2, ferrous iron (Fe^{2+}) in free hemoglobin may react with H_2O_2 as a "Fenton" reagent that catalyzes the generation of dangerous hydroxyl radicals, causing significant cellular and tissue damage (88). The in silico model of heme attack by SARS-CoV-2 implies an acceleration in the release of oxidized Hb (18), leading to acute oxidative hemolytic crisis in COVID-19 infection. In COVID-19 patients, median (IQR) serum ferritin levels for non-survivors (n=54) was extremely high at 1435.3 μg/L, compared to 503.2 μg/L in survivors (n=137) (58). High serum ferritin is often detected in patients with hemolysis (89) as iron is processed and recycled at exceedingly elevated rates during active hemolysis than under normal conditions (90). COVID-19 patients with anemia were found to have a higher mortality rate — 26% in non-survivors versus 11% in survivors (58). In a meta-analysis involving 1,210 COVID-19 patients, hemoglobin levels were found to be dramatically lower in patients with severe disease (224, 18.5%), and the decrease in hemoglobin levels was correlated to deterioration in clinical progression. (91)

3.1. Acute pulmonary embolisms in COVID-19.

Hemolysis is associated with pathologies including acute and chronic vascular disease, thrombosis, endothelial dysfunction, renal impairment/failure, immune dysregulation, and inflammation. Extravascular translocation of Hb during hemolysis can cause oxidative reactions with physiological oxidants such as hydrogen peroxide and lipid peroxides (92). Extracellular heme (ECHb) has devastating effects on the vasculature as ECHb scavenges nitric oxide (NO) to promote pathogenesis of clinical events associated with reduced NO bioavailability (83) – hypercoagulability in pulmonary hypertension where downregulation of platelet activation results in platelet aggregation and vascular clot formation. Hemolysis has another route in the promotion of thrombosis via the binding of extracellular Hb with von Willebrand factor (VWF) to competitively block cleavage by ADAMTS13 *in vitro* (93). The receptor binding of CD147 by SP may inactivate the protein, further inhibiting downstream ADAMTS13 activation (54).

Acute Pulmonary Embolisms (APE) is the third most common cause of death from cardiovascular disease after myocardial infarction and stroke (94). Pulmonary hypertension often develops after a pulmonary embolism event (95). Pulmonary hypertension and thrombotic complications are increasingly associated with hemolysis which can deplete nitric oxide by ECHb resulting in hypercoagulability (96, 83). Hypercoagulability in COVID-19 patients in a retrospective study from China has been associated with increased risk for venous thromboembolism (VTE) (97). VTE (deep vein thrombosis or pulmonary embolism) has been positively correlated with myocardial injury in that both VTE and thromboembolic arterial diseases involve the formation of clots within blood vessels (98). A study of 94 COVID-19 patients in Wuhan, China, identified significantly deranged coagulation functions, where D-dimer and fibrin/fibrinogen degradation products (FDP) were higher in patients with severe SARS-CoV-2

compared to healthy controls (99). COVID-19 medical emergencies are further complicated by diagnostic challenges presented in the overlap of symptoms in ARDS and pulmonary embolism (100). In general, patients with severe, acute pulmonary embolism (PE) have arterial hypoxemia (101).

3.2. Refractory hypoxemia and hypoxia.

COVID-19 patients under mechanical ventilation for the treatment of ARDS symptoms may experience refractory hypoxemia where adequate levels of inspired oxygen cannot compensate for inadequate arterial oxygenation. Mortality rate is extremely high from refractory hypoxemia. Physiologically, an increase in partial pressure of oxygen in arterial blood (PaO_2) of <5 mmHg if fraction of inspired oxygen (FiO_2) is increased by 0.1 is generally recognized as refractory hypoxemia (102). In COVID-19 patients, the unexpected severity of refractory hypoxemia that is dissociated from lungs exhibiting relatively normal lung functions can only be explained by acute oxidative hemolytic crisis from SARS-CoV-2 damage of erythrocytes (18), where tissue hypoxia eventually results from increased methemoglobin (MetHb) levels and the concomitant reduction of viable heme that can bind and transport oxygen and carbon dioxide.

Hypoxemia occurs when arterial oxygen tension (PaO_2) is below normal. Hypoxemia can lead to systemic hypoxia where inadequate oxygen supply restricts functions in tissues and cells. Myocardial infarction results from coronary artery obstruction leading to heart tissue ischemia. Heart failure and mortality is significantly increased by tissue necrosis from hypoxia. Myocardial injury can result from hypoxemia, where a decrease in oxygen partial pressure (pO_2) is observed (103, 104). Under normal physiological conditions, hypoxia induces gene expressions that initiate erythropoiesis, activating the maturation and proliferation of hematopoietic progenitor cells (hematopoietic stem cells) present in peripheral blood and bone marrow (105). However, it is imperative that the precise role of SARS-CoV-2 SP binding to CD147 on hematopoietic progenitor cells (HPC) be clarified as CD147 has recently been demonstrated to be important for the proliferation of HPC (106). Bone marrow and umbilical mesenchymal stem cells all express CD147 (107). It has been proposed that the loss of airway epithelial cells during COVID-19 infections is caused by defective cellular repair/renewal from viral infection of regenerative stem cells as a result of CD147/SP interactions (108, 109). The binding and membrane fusion of SP to HPC can cause membrane permeabilization, depolarization and apoptosis (110), not only in HPC, but platelets, erythrocytes and other cells that express CD147 and ACE2. Varga *et al*. introduced histological evidence of direct viral infection by SARS-CoV-2 of endothelial cells that caused extensive inflammation and apoptosis in COVID-19 patients (111).

4. VIROPORIN ION CHANNEL ACTIVITIES — INDIRECT EFFECTS OF CD147/SP INTERACTIONS

SARS-CoV-2 encodes viroporin transmembrane proteins that display ion channel activities, just like SARS-CoV-1 and other coronaviruses (112). Coronavirus viroporins are pore-forming proteins that modulate cellular ion channels in order to regulate and facilitate multiple stages of viral entry, assembly, release, and pathogenesis (113). Viroporins ORF3a and E protein in SARS-CoV-1 have been found to be indispensable for maximum replication and virulence (114, 115, 116). E proteins on the viral envelope form protein-lipid channels in cell membranes that allow passage of calcium ions, promoting virulence (117, 118). Increased intracellular calcium results in

ionic imbalance that activates the NLRP3 inflammasome, initiating release of proinflammatory cytokines (119, 120). E protein ion channel activities in SARS-CoV-1 enhanced pulmonary damage, edema accumulation and death (121). E proteins are highly conserved in coronaviruses. A functional pangenome analysis revealed that the SARS-CoV-2 E protein differs from that of SARS-CoV-1 by only three substitutions and one deletion. These substitutions and insertions are all positioned in flexible cytoplasmic regions where they would be unlikely to affect the binding proteins and ion channel functions (122). An in silico model was also able to demonstrate the similarity in functions between SARS-CoV-1 and SARS-CoV-2 E proteins, whereby the a-helix and loops present in SARS-CoV-2 E protein modulate ion channel activities, increasing pathogenesis in humans (123). The inhibition of E protein in SARS-CoV-2 has now been proposed as a viable treatment alternative for COVID-19 (122). ORF3a in SARS-CoV-1 has been shown to increase NLRP3 inflammasomes (124). Similar to the E protein, ORF3a in SARS-CoV-2 have also been found to have highly conserved domains that are linked to virulence, infectivity, ion channel formation and viral release (125). ORF3a in SARS-CoV-1 is known for its K^+ ion channel activities that cause apoptosis (126). Domain III in ORF3a of SARS-CoV-2 has been shown to consist of a K^+ ion channel (125). K+ ion channel activities from SARS-CoV-2 infection could be the reason behind hypokalemia observed in COVID-19 patients (127). Potassium ion imbalance can also cause calcium influx (128). ORF3a in SARS-CoV-1 has been shown to bind to calcium in cytoplasmic domains whereby significant changes in their protein conformation were observed upon calcium binding (129). It is of interest to note that hemolysis can also result from cellular leakage of potassium (130).

A scholarly interpretation of a paper that presented the first native RNA sequence of SARS-CoV-2 (MT007544.1) transcriptome and epitranscriptome using Oxford Nanopore Technology (131), indicated an unexpectedly high number of reads in SP for CD147 (67 reads for CD147 compared to 1 read for ACE2). The possibility that SARS-CoV-2 SP predominantly binds to CD147 over ACE2 further provides explanation of the different severe pathologies observed along with acute hemolytic crisis from erythrocyte damage.

4.1. Calcium ion influx initiates apoptotic events.

Calcium mobilization from viroporin ion channel activities after successful receptor binding creates a high gradient that allows leakages of Ca^{2+} into the cytoplasm (85). Calcium influx causes Ca^{2+} overload and subsequent collapse of ionic gradients leading to membrane permeability and apoptosis (132, 126). Calcium ion influx into endothelial cells which express CD147 (26) increased permeability and activated changes in gene expression in lung endothelial cells (133). Removal of extracellular calcium blocked the permeability increase in microvessels exposed to bradykinin (134).

4.2. Calcium overload in erythrocytes and platelets.

Calcium overload in erythrocytes contributes to hemolytic anemia through enhanced clearance from circulation (135). *In vitro* models demonstrated a dose-dependent increase in erythrocyte hemolysis with an increase in calcium concentration in medium. Inhibition of calcium influx via the use of calcium channel blockers terminated hemolysis (136). CD147 is a novel platelet surface receptor that activates platelets (55). Platelet activation is also dependent upon calcium signaling. Yet Ca^{2+} overload can lead to disturbances in platelets. Thrombosis and related disorders have

been associated with platelets with enhanced calcium influx and elevated free cytoplasmic calcium. Calcium-loaded erythrocytes adhere strongly to endothelium-derived von Willebrand factor, enhancing the formation of erythrocyte-rich thrombi (137). Patients with thromboses, hypertension, thrombocytopenia, hemolytic anemia, myelofibrosis all exhibited significant elevation of calcium levels. The use of calcium channel blocker nifedipine increased platelet count in patients with immune thrombocytopenia (138). Thrombocytopenia can also be reversed by melatonin (139) which has the ability to maintain calcium homeostasis by modulating ion channel currents, reducing calcium overload. In addition, melatonin inhibits apoptosis, protecting cells and mitochondria by increasing membrane potential, reversing the apoptotic effects of depolarization (140, 141). The potential effects of melatonin on CD147 SARS-CoV-2 Spike protein mediated damages are discussed in details below.

5. MELATONIN

Melatonin (N-acetyl-5-methoxytryptamine) is a mitochondria-targeted molecule that exists in cells of all tested eucarya and bacteria (142). Mainly synthesized in mitochondria (143), it is estimated that 99% of melatonin in vertebrates is most likely not produced in the pineal gland, nor released into the circulation upon pineal production (144). The total amount of extra pineal melatonin synthesized is estimated to exceed those in the pineal gland and in the circulation by more than two orders of magnitude, equivalent to an increase of 100-fold (145). Melatonin is a unique free radical scavenger with characteristics different from other antioxidants (146) All of the secondary and tertiary metabolites of the indoleamine can quench a diverse universe of free radicals (147). One molecule of melatonin can effectively scavenge up to ten reactive oxygen species (ROS), compared to classic antioxidants that can only neutralize one or less molecules of ROS (148). Melatonin has been extensively shown to protect mitochondria by scavenging reactive oxygen species and inhibiting mitochondrial permeability transition pore (mPTP) opening (149). The ability of melatonin to deliver systemic antioxidant protection; preserve cardiac, mitochondrial and lymphocyte functions; restore erythropoiesis; reverse hemolysis, thrombocytopenia, hypercoagulation, hypertension, and hypoxia, renders this indoleamine to be a safe and invaluable therapeutic agent during COVID-19 infections.

5.1. Melatonin protects cardiomyocyte from hypertrophy.

SARS-CoV-2 SP RBD binds to CD147 receptors that are widely expressed in cardiomyocytes (21) and endothelial cells (26). During COVID-19 infections, increased pro-inflammator cytokines can further enhance the expression of CD147 in cardiomyocytes (150). SARS-CoV-2 viroporin ion channel activities mobilize calcium ion influx into cytoplasm (117). Excessive external calcium influx increases internal calcium but depletes external serum calcium (151). Lower serum calcium is strongly correlated to sudden cardiac arrest (152). A retrospective cross-sectional study involving 241 COVID-19 patients in China found hypocalcemia (mean serum calcium levels of 2.12 (IQR, 2.04-2.20) mmol/L) in 74.7% of patients upon hospital admission. Lower calcium levels (≤2.0 mmol/L) were associated with clinical severity and prognosis, with increased organ injury, septic shock and mortality rate (153). In addition, serum levels of CD147 are often found to be highly elevated in patients with heart failure (154). It is currently unknown how SARS-CoV-2 SP binding to CD147 in cardiomyocytes affects CD147-CypA signaling which has been demonstrated to cause cardiac hypertrophy that develops into heart failure (155).

Melatonin has been demonstrated to exert therapeutic effects in the protection of cardiomyocytes from hypertrophy induced by angiotensin II (Ang II) and CD147-CypA signaling pathways. It is possible that SARS-CoV-2 SP binding to ACE2 and CD147 may disrupt Ang II and CD147-CypA activities by lowering ACE2 and upregulating CD147 to induce cardiomyocyte hypertrophy via elevated production of reactive oxygen species (ROS). Melatonin pre-treatment of H9c2 cells blocked ROS production promoted by CD147-CypA-dependent Ang II, in a concentration-dependent manner, protecting cardiomyocytes from hypertrophy (156). Although CD147 is a requisite receptor for the CypA ligand, CD147, which is highly expressed in the left ventricle (LV), has been demonstrated to promote platelet activation and pulmonary hypertension (157, 158). In a transverse aortic constriction rodent model, $CD147^{+/-}$ mice displayed less hypertrophy and less LV dilation than $CD147^{+/+}$ mice (63). The ability of melatonin to exert myocardial protection is not limited to inhibition of ROS production.

Excessive calcium influx into cardiomyocyte cytosol from possible SARS-CoV-2 ion channel activities can induce hypercontracture mediating cell death during early reperfusion after brief ischemia (159). Melatonin maintains calcium homeostasis in cardiomyocytes through modulation of IP3R and SERCA2a (Fig. 1), protecting cells against reperfusion injury *in vitro* (160). Melatonin is a mitochondria-targeted molecule that exists in every cell of every known species (142). Mitochondria provides ATP to endothelial cells while modulating intracellular calcium and the production of ROS. Survival of endothelial cells can be compromised by the opening of mitochondrial permeability transition pore (mPTP) and activation of mitochondrial apoptotic pathways (161). Increased influx of Ca^{2+} ions can suppress mitochondrial membrane potential ($\Delta\Psi m$), opening mPTP and activating mitochondrial-dependent apoptotic pathways. Melatonin, as the ancient mitochondrial-targeted molecule, protects survival of cardiac microvascular endothelial cells (CMECs) by blocking Ca^{2+} overload in mitochondria via stimulation of MAPK/ERK, inactivating CREB and inhibiting IP3R/VDAC upregulation. Melatonin preserves both structural and functional integrity of mitochondria to prevent apoptosis leading to death of CMECs (162).

5.2. ACE2 cardioprotection enhanced by melatonin.

ACE2 is highly expressed in cardiomyocytes, cardiac fibroblasts and coronary endothelial cells. SARS-CoV infections have been shown to downregulate ACE2 protein expression (5). It is currently unknown how SARS-CoV2 SP binding to ACE2 receptors may contribute to pathologies associated with ACE2 deficiency and imbalance (6-9). ACE2 is a negative regulator of the renin-angiotensin system (RAS) by converting Ang II into Ang 1-7. Loss of ACE2 increases heart failure vulnerability (8), while adequate ACE2 protected mice from ARDS associated lung injury induced by sepsis (163). The importance of nitric oxide deficiency in myocardial injury as a result of ACE2 imbalance was clearly demonstrated in an ACE2-deficient rodent model where impairment in vascular function was caused by decreased nitric oxide, and reduced eNOS expression at protein and mRNA levels (164). Nitric oxide is antifibrotic (165) and can lower blood pressure, protecting the cardiovascular system (166). Melatonin modulates blood pressure biological rhythms (167), having a profound effect on cardioprotection by reducing blood pressure and attenuating organ damage from hypertension. Melatonin regulates both central and peripheral blood pressure by increasing antioxidant defenses and modulating expression of different nitric oxide synthases (168, 169). Melatonin administered to rodents under nitric oxide depletion from L-NAME (nitric oxide synthase inhibitor) treatment, rescued animals from hypertension by lowering systolic blood

pressure, insoluble and total collagen concentration and content in the left ventricle (170). Nitric oxide deficiency during neutrophil activation can exaggerate neutrophil adhesion and endothelial cell damage (171). Under inflammatory conditions, various cell types including macrophages, dendritic cells, neutrophils, and airway epithelial cells express inducible nitric oxide synthase (iNOS) (172, 173) iNOS activation can result in the synthesis of a considerable amount of reactive nitrogen oxide species (RNOS) that mediates inflammatory processes (174). Nitric oxide derived from iNOS has been shown to cause proinflammatory and profibrotic alterations induced by particulate matter in lungs of mice (175). Melatonin is a pleiotropic immune modulator with known pro- and anti-inflammatory regulatory effects. Under inflammatory conditions, melatonin has been observed to downregulate iNOS (176). In rodent models of ventilator-induced lung injury (VILI), upregulation of IL-10 by melatonin significantly suppressed iNOS expression induced by VILI. Treatment with luzindole (melatonin receptor antagonist) counteracted the protective effect of melatonin (177).

5.3. Melatonin rescues neutrophil net induced vascular permeability and hypercoagulation.

One theory identified neutrophil extracellular traps (NETs) as a possible cause for hypercoagulation activities in severe COVID-19 patients. High blood levels of NETs, composed of extracellular DNA fibers that bind pathogens, are correlated with thrombin levels that predict adverse cardiac events that may lead to major organ damages in lungs, heart and kidneys (178). A retrospective analysis of 452 COVID-19 patients found a significant elevation of neutrophil counts (4.3 vs 3.2 $\times 10^9$; $P < 0.001$) in severe cases (42). NETs are able to modify endothelial barrier structures, causing vascular endothelial permeability that reduces anti-thrombotic and anti-inflammatory features (179, 180). An *in vivo* rodent experiment demonstrated that local injection of melatonin (14 or 140 pg) could effectively reduce endothelial cell vascular permeability induced by leukotriene B4-activated neutrophils. The reduction in vascular permeability was likely mediated through the inhibition of endothelial cell hyper-adhesiveness by melatonin (181). In humans, oral melatonin administration (3 mg) resulted in an inverse relationship with procoagulant measures in 46 healthy young men, where increased plasma melatonin predicted lower levels of FVIII:C ($P = 0.037$) and fibrinogen ($P = 0.022$) (182). Hypercoagulability and thrombotic complications are frequently observed in patients with hemolytic anemia (183).

5.4. Melatonin attenuates hypoxemia from nitric oxide dysregulation in oxidative hemolysis.

Hypercoagulability and thrombosis are frequently associated with increased hemolysis as a result of nitric oxide depletion via several mechanisms. Compartmentalized hemoglobin within erythrocytes or haptoglobin have limited ability to scavenge nitric oxide (NO) (184). *In vitro*, NO reaction time with free hemoglobin (Hb) to form Hb-NO products is 1,000-fold faster than with erythrocytes (185). Vasoconstriction and coagulopathy from the dysregulation of NO during hemolysis is exacerbated by the release of arginase which converts L-arginine into ornithine, further diminishing NO production by reducing NO substrate L-arginine (186). Platelets are an important source of NO. Platelet dysregulation from CD147/SP interactions on platelet membranes can cause NO dysregulation, facilitating platelet activation, adhesion, and aggregation that eventually results in arterial thrombosis (187). Nitric oxide gas inhalation to increase NO availability is now under clinical trial as possible treatment for COVID-19 (188). The use of inhaled nitric oxide should be carefully monitored in the context of oxidative hemolysis due to the

possible induction of diffusion hypoxemia, commonly known as the "Fink" or "Third Gas Effect" where PaO_2 can be significantly lowered when O_2 is below 21% saturation (189). A study discovered an alarming correlation between long-term nitrogen dioxide (NO_2) and COVID-19 fatality where 83% of fatalities (3,701/4443 cases) examined occurred in areas where maximum NO_2 concentration was above 100 μmol/m2, while only 1.5% (51/4443 cases) of all COVID-19 fatalities occurred in areas where maximum NO_2 concentration was below 50 μmol/m2 (190). Higher concentration of NO delivered during inhaled NO therapy may also shift the oxygen dissociation curve to the left as nitric oxide increases oxygen affinity of Hb, decreasing heme-heme interactions and increasing formation of MetHb while further reducing oxygen-carrying capacity of hemoglobin in blood (191-193). Yet nitric oxide can exert allosteric effects on Hb to either increase or decrease its affinity to oxygen through different biochemical reactions that yield different Hb products. Increased Met-Hb and SNO-Hb shifts the oxygen dissociation curve towards the left, while increased $HbFe^{2+}NO$ shifts the curve the opposite direction to the right (194). Hb-NO product formed as a result of NO dysregulation and subsequent reactivity with target molecules such as Hb can exacerbate endothelial dysfunctions and associated myocardial injury in COVID-19 patients (195). Optimal regulation of NO production and curtailing downstream effects from NO dysregulation is best achieved through the use of melatonin.

Melatonin is an ancient pleiotropic molecule that can regulate NO dysregulation achieving different biological effects under different situations. Melatonin is well known for its circadian antihypertensive effects. Gene chip analysis of umbilical endothelial cells after 6 hours of melatonin treatment revealed 121 upregulated genes and 214 downregulated genes that resulted in a significant elevation of NO levels and endothelial nitric oxide synthase (eNOS) activity, together with a decrease in endothelin and Ang II (196). While in rodents with metabolic syndrome, melatonin treatment (10 mg/kg/day for 3 weeks) effectively decreased blood pressure by upregulating nitric oxide synthase (eNOS) protein expression and activities in the brain but not the heart (197). On the other hand, under hypobaric hypoxic conditions where rats were exposed to partial oxygen concentration similar to being at 9000 m elevation, pretreatment with melatonin (100mg/kg) inhibited hypoxia-induced increase of nNOS, eNOS, iNOS that would have resulted in neuropsychological dysfunctions including insomnia, dizziness and memory deficiencies (198).

Increasing evidence showed cumulative incidence of venous and arterial thrombotic complications among COVID-19 patients admitted to ICU, with pulmonary embolism (PE) being the most common insult (199). Most patients with severe, acute pulmonary embolism (PE) have arterial hypoxemia (101). Hypoxemia can lead to systemic hypoxia where inadequate oxygen supply restricts functions in tissues and cells, leading to pulmonary hypertension and vascular remodeling. Daily administration of melatonin (10mg/kg) to hypoxic rats attenuated pulmonary hypertension and vascular remodeling by dramatically increasing eNOS phosphorylation in the lung that restored NO production (200). Hypoxia not only affects NO homeostasis (201), it can alter erythrocyte membrane protein band 3 to initiate hemolysis (202).

6. MELATONIN PROTECTS ERYTHROCYTES FROM OXIDATIVE HEMOLYSIS

Erythrocytes are able to neutralize endogenous and exogenous reactive oxygen species (ROS) using a robust antioxidant system. Under normal conditions, methemoglobin (MetHb) – hemoglobin that is oxidized into the ferric (Fe^{3+}) form, unable to bind and transport oxygen – is reduced back to hemoglobin by NADH–cytochrome b5–metHb reductase. Although Hb auto-oxidation rate is quite slow at around 0.5% to 3% daily, the process generates intracellular ROS

H_2O_2, where insufficient intracellular reducants (glutathione, catalase) can cause oxidative damage to membranes via lipid peroxidation (203). The formation of ferryl hemoglobin and oxoferry hemoglobin when intracellular H_2O_2 oxidizes Hb can cause hemoglobin denaturation in erythrocytes (204). Oxidative denaturation of hemoglobin produces membrane damage via the release of hemin that results in the alteration of structural and functional properties of membranes, leading eventually to the destruction of erythrocytes (205). Addition of melatonin *in vitro* to erythrocytes exposed to oxidative stress that would have resulted in 100% hemolysis in 180 minutes, inhibited the formation of protein carbonyls and hemin precipitation for 20 mins and 10 mins respectively. During oxidative hemolysis, melatonin was observed to be rapidly absorbed into the cytosol and consumed by erythrocytes, delaying Hb denaturation and release of hemin (206).

Melatonin can effectively reduce both ferryl hemoglobin (Fe^{4+}= O^{2-}) and oxoferryl hemoglobin, the Hb oxidation products that cause Hb denaturation and heme destruction, back into MetHb in the ferric (Fe^{3+}) form (207, 208). Heme in the ferric (Fe^{3+}) form has been shown to dissociate from Hb at much higher rates than in the ferryl (Fe^{4+}) form, as MetHb is less stable due to the occupancy of water coordinated to the iron ion (87). Yet ferryl hemoglobin is highly oxidative as it is associated with radical proteins that can cause lipid peroxidation and generate cytotoxic bioactive molecules (209). The timely reduction of MetHb into functional heme in the ferrous (Fe^{2+}) form by NADH–cytochrome b5–metHb reductase is essential in the prevention of rapid heme loss (203).

6.1. Melatonin recycles NAD^+ to maintain NADH-cytochrome b5 reductase.

90% of heme degradation products in hemolysis are found on membranes of erythrocytes (204). The integrity of the antioxidant redox system such as NADH–cytochrome b5 reductase (NADH–cytochrome b5–metHb reductase) on membranes preserves Hb functionality and survival. Patients with recessive hereditary methemoglobinemia have been associated with deficits in this enzyme (210-212). The human CYB5R3 gene encodes two forms of NADH-cytochrome b5 reductase. Only the erythrocyte-restricted soluble form that is bound tightly to the inner face of the erythrocyte membrane has been shown to be active in methemoglobin reduction (213). The ubiquitous membrane-associated form involved in lipid metabolism is expressed in subcellular compartments such as the endoplasmic reticulum, the mitochondrial outer membrane, and the plasma membrane (214). NADH-cytochrome B5 reductases are the default mechanisms responsible for the reduction of MetHb back into functional heme in the ferrous (Fe^{2+}) form in erythrocytes (203), using NADH as principal reductant. All CYB5R enzymes use NADH as electron donors in one-electron transfer cellular reduction-oxidation reactions (214). The ability of melatonin to recycle NAD is perhaps one of the main reasons why this indoleamine has been found to be actively utilized by erythrocytes under oxidative stress conditions, protecting erythrocytes from hemolysis as a result of hemoglobin denaturation and the release of free heme (206).

Melatonin is an ideal electron donor due to its electron-rich aromatic indole ring (215) Melatonin has been demonstrated in both cell-free and cultured PC12 cells to preserve NADH levels under oxidative stress where melatonin readily donates an electron to reduce the NAD radical (216). By recycling NAD^+, the oxidized form of NAD, melatonin maintains a constant, adequate, intracellular supply of NADH to support the reduction of MetHb by NADH-cytochrome b5 reductase. Since erythrocytes are highly sensitive to oxidative stress, the fact that the active metabolites generated as a result of melatonin oxidation such as N^1-acetyl-N^2-formyl-5-

methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK) are also effective free radical scavengers, renders this electron-rich indolamine to be a unique antioxidant (217).

The protective role of melatonin in blood may also be linked to its association with a class of two-electron quinone reductase called NRH:quinone oxidoreductase 2 (NQO2). The NQO gene encodes NQO1 and NQO2 — the two major quinone reductases in mammalian systems (218). NQO1 is not expressed in blood under normal conditions (219), whereas NQO2 is expressed at higher levels in blood (220). The nomenclature of NQO1 and NQO2 may sometimes be confused with NADH-cytochrome b5 reductase due to the common use of the term 'diaphorase' for both classes of enzymes more than 50 years ago (221). The catalytic activity of NQO2, a known human target of the antimalarial drug chloroquine (CQ), can be inhibited by binding within active sites to chloroquine, resveratrol, and melatonin (222, 223). Even though NQO2 has been regarded by some as the third binding site for melatonin (MT3) (224), its affinity to the enzyme is much lower when compared to potent flavonoid inhibitors like quercetin and resveratrol (225). Flavonoids are not endogenous inhibitors of NQO2. Plants that produce flavonoids have not been identified with NQO genes, whereas vertebrates expressing NQO genes synthesize melatonin but not flavonoids (221). It is possible that the inhibition of NQO2 by melatonin is meant to occur only under specific conditions as melatonin has been shown to bind to only the free oxidized (FAD) form of NQO2 (223), and NQO2 is known for its ability to generate reactive oxygen species such as superoxide (226, 227). The discovery in 2017 by Cassagnes *et al*. that anti-malarial indolone-type derivatives could serve as substrates of human NQO2 instead of acting as inhibitors such as chloroquine (228), revives the hypothesis that melatonin could be a naturally occurring co-substrate for NQO2 (229). This hypothesis could not be confirmed by another group employing nuclear magnetic resonance studies (230). In view of the significance of melatonin as a potential treatment for COVID-19, further studies on the relationship between melatonin and NQO2 are necessary.

6.2. Melatonin protects Band 3 membrane protein in erythrocytes.

Band 3 protein is the essential anion exchanger on erythrocyte membranes responsible for ion balance, maintaining the shape and integrity of erythrocytes, and optimizing oxygen delivery to cells and tissues (231, 232). The Band 3 glycoprotein or anion exchanger 1 (AE1) encoded by SLC4A1, plays a critical role in efficient respiration by facilitating the electroneutral exchange of chloride and bicarbonate across erythrocyte plasma membranes (231). Band 3, being the most abundant protein (about 25%) in the erythrocyte plasma membrane, is responsible for the largest ion-specific flux known for any single cell in the body (233). Carbon dioxide is converted into bicarbonate after it is diffused into erythrocytes. Band 3 protein transports the bicarbonate anion out of the cell in exchange for a chloride anion. This exchange process is reversed when Band 3 transports the bicarbonate anion into the cell in exchange for a chloride anion. The bicarbonate anion is then converted into carbon dioxide which will diffuse out of the cell to be expelled by the lung (231). A hypoxic microenvironment can upregulate genetic expression of CD147 via HIF-1a mediated transcription factors (234). Elevated intracellular calcium as a result of CD147/SP interactions induces band 3 protein tyrosine phosphorylation promoted by dissociation of protein tyrosine phosphatase (PTP) from band 3 (235). Protracted oxidative stress-induced phosphorylation of band 3 initiates a series of events that eventually results in oxidative hemolysis (236, 237). *In vitro*, melatonin at 100 μM concentration protects band 3 protein from H_2O_2-induced oxidative damage to membrane conformation and arrangement, preserving erythrocyte morphology integrity supporting deformability and cell shape to allow for optimal gas diffusion

(238-240). Melatonin has been observed to protect band 3 protein even in the absence of catalase, restoring antioxidant enzyme activities in erythrocytes challenged by severe oxidative stress (241). During oxidative hemolysis, heme is the major source of reactive oxygen species (ROS). Free heme can become a potent cytotoxic pro-oxidant due to the iron (Fe) atom contained within its protoporphyrin ring. Hemolytic products including heme and hemin have been shown to directly target the endothelium to promote endothelial cell pro-inflammatory activation in the lung (92, 242-244). Free iron-protoporphyrin in hemin liberated during hemolysis, can promote lipid peroxidation. It has been suggested that endothelial cell death and vascular dysfunction may be the result of mitochondrial dysfunctions caused by posttranslational modification of proteins generated by lipid and reactive oxygen species produced by hemin during hemolytic events (245). A 'multiple-hit' model described how hemolysis initiated by a pathological event can release toxic products that are potent endothelial stressors leading to the development of proinflammatory and prothrombotic environments resulting in endothelial cell damage or cell death (246). SARS-CoV-2 is now believed to induce endotheliitis in multiple organs as viral elements have been identified in endothelial cells that exhibited high levels of inflammation and cell death (111). Children infected by COVID-19 have been found to display pediatric hyperinflammatory syndrome with symptoms similar to Kawasaki disease (247) Kawasaki disease is strongly associated with endothelial cell damage and dysfunction (248-251). Melatonin may attenuate oxidative damage to erythrocytes during hemolysis by protecting Band 3 protein, and chelating free iron to inhibit iron overload in erythrocytes, hepatic, and renal tissues. Even though chelation of iron by melatonin has been reported in literature (252, 253), the exact mechanisms involved remain unclear.

6.3. Melatonin preserves endothelial cells calcium homeostasis during hypoxia.

Viroporin-induced ion channel activities as a result of CD147 receptor binding to spike protein can increase Ca^{2+} influx, initiating oxidative hemolysis (26, 126, 132, 136). Hypoxia as a result of hemolysis in turn exacerbates influx of Ca^{2+} in endothelial cells (254). Calcium overload resulting in oxidative stress-mediated impaired Ca^{2+} handling in the sarcoplasmic reticulum (SR) leads to deterioration of myocardial functions. Rodents exposed to 10% oxygen for 4 weeks but injected with melatonin showed elevated cardioprotection against myocardial injury under hypoxia by preserving sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) expression, thus improving SR Ca^{2+} handling and calcium homeostasis (255). Hypoxia can increase HIF-1α-mediated migration speed of endothelial cells by 1.4-fold (256). Melatonin downregulates HIF-1α signaling pathways (ERK/Rac1), suppressing HIF-1α expression and stabilization to prevent endothelial cell migration under hypoxic conditions (257, 258). Suppression of HIF-1α expression and stabilization can also reduce increased CD147 activation under hypoxic conditions (234), possibly reducing SARS-CoV-2 virulence and replication. Calcium mobilization can activate NLRP3 inflammasomes, leading to cytokine storms (120). Extracellular Hb from oxidative hemolysis has been demonstrated to activate NLRP3 inflammasomes, initiating platelet activation, aggregation and consumption, resulting in platelet derangement (68, 69, 72). ECHb also enhances VWF platelet adhesion, disturbing the balance between coagulation and fibrinolysis (75). NLRP3 inflammasome is a known target of melatonin (259). Melatonin inhibits NLRP3 inflammasomes through enhancement of Nrf2 expression (260). Melatonin supplementation may reduce inflammatory effects of cytokine storms induced by SARS-CoV-2 infection by reversing aerobic glycolysis in immune cells (261). The ability of melatonin to increase erythropoiesis, protect platelets and lymphocytes, as well as its capacity to regenerate NADH supplying essential electrons to protect

erythrocytes against oxidative hemolysis as a result of CD147/SP interactions, further supports its use during COVID-19 infections *(*206, 216).

7. MELATONIN ENHANCES ERYTHROPOIESIS, PROTECTS LYMPHOCYTES AND PLATELETS

Melatonin has been shown *in vitro* to be readily absorbed by erythrocytes via GLUT1 transporters. The addition of glucose to the growth medium can markedly enhance uptake (262). Melatonin's association with glucose is closely related to its ability to enhance the expression of glucose-6-phosphate dehydrogenase (G6PD). G6PD is the rate-limiting enzyme in the pentose phosphate pathway (PPP). The PPP consists of an oxidative phase that generates NADPH and a nonoxidative phase that interconverts phosphorylated sugars (263). NADPH produced by G6PD in the PPP is required for the regeneration of oxidized glutathione (GSSG), Without adequate reduced glutathione (GSH), hemoglobin cannot be maintained in its soluble form under oxidative stress (264). Hemolytic events can be easily triggered under conditions of G6PD deficiencies (265, 266). Oral and IV glutathione treatments given to COVID-19 pneumonia patients have been shown to substantially alleviate dyspnea and respiratory distress (267).

7.1. Melatonin upregulates G6PD activity during erythropoiesis.

G6PD is essential during differentiation of hematopoietic stem cells, protecting these progenitors from oxidative stress (268, 269). During erythropoiesis, after erythroblasts switch to Hb composition during late maturation (270), definitive erythrocytes after hemoglobin switch can undergo apoptosis in mice deficient in G6PD enzymes. Apoptosis of definitive erythrocytes can only be prevented by the restoration of G6PD activity which is indispensable for definitive erythropoiesis (271). Melatonin at pharmacological doses has been shown in both *in vitro* (0.08 mM to 0.1mM) and *in vivo* (10 mg/kg) animal models to markedly enhance G6PD enzyme activity in human erythrocytes and in Sprague-Dawley type rats respectively (272). In animal models of lead-induced suppression of the heme synthesis pathways, daily intragastric pretreatment with melatonin (30mg/kg) prevented lead toxicity-induced inhibition of heme-synthesizing enzymes and iron deficiency, restoring enzymatic and non-enzymatic antioxidant levels, while normalizing zinc and copper levels in the liver (273). Anemic patients with chronic kidney disease exhibit erythropoietin (EPO) hyporesponsiveness where there is a significant decrease in Hb or failure to raise Hb despite adequate EPO dose (274). Erythropoietin hyporesponsive anemic patients receiving 5 mg of melatonin showed an impressive increase in Hb, serum iron, and transferrin saturation ratio (TSAT) compared to baseline. When compared to control groups and baseline, patients receiving melatonin displayed significantly reduced inflammatory biomarkers (275).

7.2. Melatonin restores normal lymphocyte functions.

COVID-19 non-survivors have significantly lower lymphocyte counts than survivors, as functional exhaustion of lymphocytes is correlated to disease progression (40, 41). Glutathione is required for T-lymphocyte proliferation. Inhibition of glutathione can reduce T-lymphocyte DNA synthesis by more than 75% (276). T-lymphocytes produce glutathione using the pentose phosphate pathway where G6PD is the rate-limiting enzyme (277). Glutathione has also been shown to increase production of IL-2 in human lymphocytes. (278). Resting and

phytohemagglutinin-stimulated human lymphocytes have been demonstrated to naturally produce and release high amounts of melatonin that exceed nocturnal human physiological levels in serum of up to five-fold. Inhibition of the melatonin biosynthetic pathways in lymphocytes led to the decrease in IL-2 production, which could be reversed by addition of exogenous melatonin *in vitro* (279). IL-2 is generally regarded as a pro-inflammatory cytokine (280). There are indications that the production of IL-2 during viral infections can exacerbate the inflammatory environment in the lung (281). The production of IL-2 by lymphocytes may serve to promote effector CD4 T cell long-lived memory generation following pathogenic challenges. Deficient production of IL-2 by effector CD4 T cells initiates default apoptosis, while the addition of exogenous IL-2 can directly rescue some $CD4^+$ from acute apoptotic death (282, 283). Melatonin is a multi-tasking pleiotropic immune modulator with known pro- and anti-inflammatory regulatory effects, with the capacity to reduce IL-2 expression and release during severe inflammatory conditions (284). Melatonin synthesized by lymphocytes not only regulates the IL-2/IL-2 receptor system (285), but could be essential in ensuring survival and functioning by enhancing G6PD enzyme activity, protecting glutathione levels (272).

7.3. Melatonin attenuates thrombocytopenia from oxidative hemolysis.

The CD147CypA signaling pathway activates platelets leading to enhanced adhesion and thrombosis (57). Plasma levels of soluble CD147 (sCD147) in healthy donors and patients with or without coronary artery disease were found to be significantly correlated with standard platelet biomarker soluble GPVI (sGPVI, r=0.46, p=.004). sCD147 levels can be predicted using linear regression analysis of sGPVI levels ($\beta = .445$, $p = 0.003$) and age ($\beta = 0.304$, $p = 0.038$) (157). The effects of CD147/SP interactions on platelets needs to be further clarified as 20.45% +/- 1.63% of circulating platelets express CD147 in humans (56). Platelets contain whole, functional, respiratory competent mitochondria in normal physiological state (286). During oxidative hemolysis, oxidized cell-free MetHb can rapidly deplete platelets via the activation of ROS-mediated mitochondrial apoptotic pathways (287). Antioxidant protection from oxidative stress is essential for the survival and functionality of platelets. Patients with known G6PD deficiency showed enzyme activity reduced to 15% of control subjects, together with greatly diminished NADPH and glutathione levels, and elevated platelet aggregation measurements (288). In an irradiated mouse model (4 Gy), mice receiving 10mg/kg intraperitoneal injection of melatonin for 21 consecutive days after irradiation displayed enhanced recovery rate of platelets and white blood cell counts compared to controls without melatonin treatment (289). In human subjects, 200 patients with persistent thrombocytopenia due to different causes were randomized to receive 20mg/day melatonin orally in the evening for one month. 71/98 (72%) of patients achieved normalization of platelet counts where mean platelet number was rapidly and significantly increased. Among the patients, thrombocytopenia from disseminated intravascular coagulation (DIC) had the lowest response rate to melatonin supplementation (290). At present, the full implications of SARS-CoV-2 spike protein binding with CD147 and the subsequent effects on CD147-CypA signaling are not completely understood. Cyclophilin A has been associated with the pathogenesis of viral infection and replication including SARS-CoV (291, 292). The fact that CyPA is also identified as a target for antiviral therapy (293) warrants further exploration into its role during COVID-19 infections.

For the best interests of readers, the potential mechanisms of melatonin targeting CD147 SARS-CoV-2 spike protein are summarized in Figure 1.

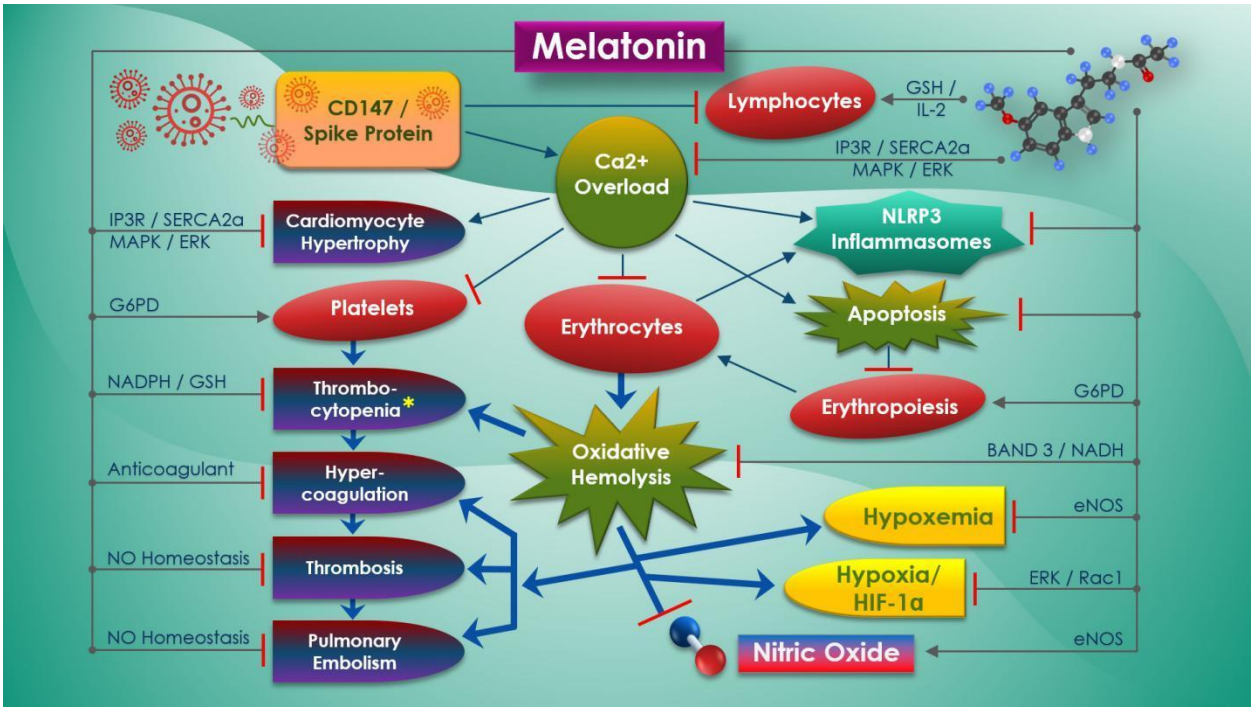

Fig. 1. The potential mechanisms of melatonin in the attenuation of CD147 SARS-CoV-2 spike protein mediated damages during infection.

**COVID-19 patients may show elevated von Willebrand factor and factor VIII, fibrinogen and fibrinogen degradation products D-dimer, while platelet counts are depleted, normal or elevated. GSH: glutathione; NO: nitric oxide; Ca2+: calcium ions; G6PD: glucose-6-phosphate dehydrogenase; eNOS: endothelial nitric oxide synthase; arrows: stimulations; red bars: attenuation.*

8. CONCLUSION

Melatonin is a well-established, potent, free radical scavenger with pleiotropic functions that includes epigenetic regulation (294) affecting circadian biology and maintenance of systemic homeostasis of essential biological processes. The ability of melatonin to preserve the survival and functioning of erythrocytes, platelets, lymphocytes, leukocytes, and mitochondria during SARS-CoV-2 infections renders this ancient molecule to be an indispensable component in the arsenal of pharmacological and natural remedies in the attenuation of symptoms in COVID-19 patients suffering from hypoxemia, myocardial injury, and other hemoglobinopathy related pathologies caused by oxidative hemolysis induced by CD147/SP interactions during receptor binding and subsequent viral fusion and invasion. Furthermore, melatonin can exert a formidable range of influences over the human immune response system, as discussed in great detail by Tan and Hardeland (295), as well as Anderson and Reiter (296). The need to identify optimal dosages for melatonin as effective treatment for COVID-19 infection is an urgent priority (297). While further clarification of CD147/SP interactions and effects is urgently needed, this review has demonstrated the efficacies in the use of melatonin as a safe and effective treatment for the prevention and attenuation of unique pathologies that may be related to CD147/SP interactions during COVID-19 infections.

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AUTHORSHIP

DL wrote and edited this article.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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