Review

Mitochondrial dynamics in myocardial ischemia/reperfusion injury: Effects of melatonin

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ABSTRACT

Timely reperfusion during myocardial infarction paradoxically leads to ischemia/reperfusion (I/R) injury. Mitochondrial quality control has emerged as a key participant in regulation of this process. The aims of this review are to briefly summarize current evidence for the role of mitochondrial quality control in I/R injury and to evaluate whether the cardioprotective actions of melatonin, a potent free radical scavenger and antioxidant, can be attributed to its effects on these processes. Using a variety of experimental models, *in vivo* and *in vitro*, melatonin-induced cardioprotection has been demonstrated to be associated with attenuation or reversal of the harmful effects of I/R on parameters of mitochondrial quality control as evidenced by (i) upregulation of mitochondrial fusion and inhibition of fission, particularly Drp1 expression and translocation from the cytosol to the mitochondria; (ii) changes in both the conventional and alternative mitophagy pathways. Melatonin significantly upregulates mitochondrial biogenesis and the expression of sirtuins 1, 3 and 6 and has a beneficial effect on mitochondrial-endoplasmic reticulum interaction in I/R. A novel observation is the ability of melatonin to stimulate intercellular transfer of mitochondria between damaged cells, although this has not yet been demonstrated in myocardial I/R. Melatonin treatment has profound effects on the diabetic heart: it reverses the significant reduction in function and inhibits the progression of diabetic cardiomyopathy which was associated with a significant effect on mitochondrial quality control, as evidenced by a reduction in fission. In summary, available evidence supports a major role for mitochondrial quality control in the beneficial actions of melatonin in the I/R heart.

Key Words: melatonin; mitochondria quality control; fission; fusion; mitophagy; biogenesis; cardiac ischemia and reperfusion.

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1. INTRODUCTION

Ischemic heart disease, and more specifically myocardial infarction (MI), is still the leading cause of morbidity and mortality in developed countries (1). It is well-established that timely reperfusion during myocardial infarction is crucial for the salvage of the ischemic myocardium,

but this paradoxically leads to ischemia-reperfusion injuries which cause the final myocardial damage (2). Restoration of blood flow (reperfusion) by, for example, primary percutaneous coronary intervention or thrombolytic drugs aggravates tissue damage due to several pathophysiological mechanisms (3). In particular, this is a consequence of the exacerbated generation of reactive oxygen and nitrogen species (ROS/RNS) that induces further cardiomyocyte death and affects other cell types such as fibroblasts, endothelial cells or smooth muscle cells (3,4). Intensive efforts to clarify the precise cellular and molecular mechanisms in the ischemic/reperfused heart are reflected by the many comprehensive reviews on the topic (see for example references 2, 4-6).

The mitochondrion has emerged as a key participant in the regulation of myocardial injury during myocardial ischemia/reperfusion (I/R) (6–9). Mitochondria represent about one third of the cardiac mass and are responsible for energy production via the oxidative phosphorylation process. Loss of function, as occurs when the heart is exposed to low oxygen levels, causes mitochondrial damage. Several mechanisms have been postulated for this phenomenon including mitochondrial Ca^{2+} overload, ROS generation, autophagy failure, platelet activation, and micro-thrombosis formation (5, 10). Recent attention also focused on the concept that changes in, or an imbalance of mitochondrial dynamics/morphosis, are critical in the development of I/R -induced cardiomyocyte death (7,11–17).

Our aims in this review are firstly to summarize current evidence regarding the role of mitochondrial quality control (including mitochondrial morphosis and mitophagy) in I/R-induced cardiomyocyte death. Secondly, the possibility will be discussed whether the cardioprotective actions of melatonin, a well-established scavenger of free radicals and an antioxidant, can be attributed to its effects on mitochondrial quality control processes.

2. MITOCHONDRIAL QUALITY CONTROL: FUSION AND FISSION

Balanced dynamics are essential to adjust mitochondrial metabolism to meet the energy demands of cardiomyocytes (7, 18, 19). Quality control processes are largely regulated by mitochondrial dynamics and mitophagy, through processes such as fusion and fission. There is growing evidence that dysregulation of fission-fusion contributes to the maladaptive changes underlying the fate of the myocardial cell in stress conditions and several reviews on the significance of mitochondrial dynamics in myocardial I/R have been published recently (11–15). Mitochondria undergo fission to generate fragmented discrete organelles which are required for cell division and removal of damaged mitochondria by the process of mitophagy. On the other hand, mitochondrial fusion is a protective mechanism that repairs damaged mitochondria and improves the stability of mitochondrial DNA by fusing poorly structured mitochondria with the network of healthy mitochondria.

It is now known that mitochondrial fusion is orchestrated by the GTPases optic atrophy 1(OPA1) and mitofusins 1 and 2 (Mfn1, Mfn2) that lead to elongated mitochondria and exchanges of mitochondrial matrix proteins and mitochondrial DNA. Mfn1 and Mfn2 mainly mediate fusion of the outer mitochondrial membranes (OMM), while OPA1 plays an important role in fusion of the inner mitochondrial membranes. OPA1 is also critical for cristae morphogenesis and maintenance of their architecture, regulation of respiratory supercomplex assembly and tethering of cytochrome c within the mitochondrial cristae (20–23). While OPA1 acts in concert with Mfn1 (but not Mfn2) to achieve fusion of the inner mitochondrial membrane (IMM), maintenance of cristae architecture is strictly reliant on OPA1 GTPase activity (24, 25). Given that a reported 80%

of cytochrome c is bound within the cristae by OPA1, apoptosis is highly dependent on IMM architecture and OPA1 integrity (26, 27).

In contrast, fission results in smaller fragmented mitochondria, with the GTPase dynamin related protein (Drp1) as an important role player. Fission is required to selectively remove damaged mitochondria by mitophagy (which are then replaced by mitochondrial biogenesis) and allows fragmented mitochondria which are still viable to re-enter the mitochondrial network. Drp1 translocates from the cytosol to the outer mitochondrial membrane under certain conditions such as stress. Drp1 oligomerization leads to mitochondrial fission via its interaction with outer membrane proteins, including fission factor 1 (Fis1), mitochondrial fission factor (Mff) mitochondrial division protein 1(Mdv1) and mitochondrial dynamics proteins of 49 and 51KDa (MiD49 and MiD51) (28). Drp1 translocation to the mitochondria promotes Bax/Bak recruitment, oligomerization and pore formation at the OMM, thereby triggering cytochrome c release and apoptosis (29, 30).

Although the specific role of each of the adaptor proteins in the fission process is not clear yet, it has been suggested that Fis1 is responsible for Drp1 recruitment during pathological fission whereas Mff and MiD49/51 assume a predominant role during physiological fission(31–34) However, previous studies from Zhou and coworkers (35) reported that Mff was increased in reaction to I/R injury and involved in Drp1-required mitochondrial fission. The exact mechanisms underlying Mff-upregulation remain unclear. These workers (36) investigated the role of the dual-specificity phosphatase (DUSP) in this regard and found that I/R injury caused significant downregulation of this phosphatase, leading to an increase in JNK phosphorylation. This, in turn, increased the expression of Mff initiating fatal mitochondrial fission as well as increased phosphorylation of Bnip3 and elevated mitophagy. It was suggested that DUSP probably lies upstream of Mff-dependent mitochondrial fission in the I/R heart.

3. THERAPEUTIC TARGETING OF FUSION AND FISSION PROTEINS FOR CARDIOPROTECTION

The potential for therapeutic targeting of mitochondrial fusion and fission proteins has elicited considerable interest and was recently reviewed by Hernandez-Resendiz and co-workers (37). Interpretation of the studies on fusion proteins is quite challenging given the pleiotropic non-fusion functions of Mfn2 and OPA1, which may also contribute to cardioprotection. For example, deletion of both Mfn1 and Mfn2 in the adult heart inhibited the mitochondrial permeability transition pore (mPTP) opening and reduced myocardial infarct (MI) size (38). This cardioprotective effect was attributed to the pleiotropic non-fusion role of Mfn2 in tethering of mitochondria to the sarcoplasmic reticulum to create localized microdomains through which calcium could transit (39, 40). Therefore, targeting Mfn2 during acute myocardial I/R to transiently disassociate mitochondria from the SR may provide a novel cardioprotective strategy.

Interpretation of the cardioprotective effects of OPA1 is also challenging due its potential non-fusion effects on mitochondrial cristae remodelling that prevent apoptosis, facilitate the assembly of electron transport supercomplexes and improve mitochondrial respiratory efficiency. In terms of therapeutic targeting of OPA1 as a cardioprotective strategy, a recent study has identified epigallocatechin gallate as a novel pharmacological inhibitor of OMA1 (a protease responsible for cleaving L-OPA1 to S-OPA1). Treatment of mouse embryonic fibroblasts with epigallocatechin was able to prevent cleavage of L-OPA1 to S-OPA1, inhibit mitochondrial fission, prevent apoptosis and reduce cell death following simulated I/R, providing a potential therapeutic strategy

for reducing MI size (41). However, although these studies implicate the mitochondrial fusion proteins, Mfn2 and OPA1, as potential targets for cardioprotection, the mechanisms underlying their beneficial effects appear to be related to their non-fusion pleiotropic functions rather than their pro-fusion effects.

Inhibition of fission during reperfusion appears to have more cardioprotective potential. Excessive mitochondrial fission is known to be detrimental to the heart. For example, excessive mitochondrial fission occurs within 60 min. of myocardial reperfusion after transient ischemia, leading to mitochondrial dysfunction and decreased cardiac contractility (42). Ser 637 of Drp1 is dephosphorylated during reperfusion, which in turn induces its translocation from the cytosol to the mitochondria (43) and increases mitochondrial fission. Disatnik and coworkers also showed that treatment of the heart with selective inhibitors of Drp1, including Mdivi-1 and P110, a peptide inhibitor that inhibits the interaction between Drp1 and hFis (42), protects the heart against I/R injury (14, 42).

The Drp1 inhibitors preserve mitochondrial morphology, decrease cytosolic calcium, inhibit mPTP opening and prevent apoptosis in cardiomyocytes, thereby decreasing myocardial injury (13, 42). Other pharmacological inhibitors of mitochondrial fission, such as Dynasore (a non-specific inhibitor of dynamins), have also been shown to be cardioprotective (44).

Recent studies suggest that Mdivi-1 has off-target effects that are independent of its inhibitory effects on Drp1 GTPase activity, for example, it is a weak and reversible inhibitor of complex I and complex II (44). However, the significance of Drp1 inhibition in cardioprotection was indicated by the finding that two novel Drp1 inhibitors (Drpitor1 and Drpitor1a), shown to be more potent and specific than Mdivi-1 in terms of inhibiting Drp1 GTPase activity (without complex I inhibition), also conferred cardioprotection against acute I/R in the isolated perfused rat heart when given as a pre-treatment (45).

While the above studies implicate I/R-induced mitochondrial fission as a critical mediator of cardiomyocyte death following acute myocardial infarction (AMI) that can be targeted indirectly via a number of cardioprotective agents, other studies have shown that Drp1 is important in maintaining mitochondrial homeostasis under some conditions (44, 45). For example, during exercise mitochondrial fission enhances, rather than impairs, mitochondrial function (46) while persistent inhibition of Drp1 by genetic or repetitive application of Mdivi-1 in the heart exacerbates I/R injury (46). Thus, it is likely that although dysregulated activation of Drp1 in response to stress is detrimental for the heart, persistent inhibition of Drp1 may also be detrimental. However, since Drp1 may have fission-independent functions (44), and given that chemical inhibitors of Drp1 appear to have some Drp1-independent effects (47), further investigation is required to clarify whether cardiac dysfunction is induced primarily by excessive fission and whether other molecules besides Drp1 are also involved in its pathogenesis.

4. MITOPHAGY

A subsequent component of mitochondrial quality control is mitophagy, the selective form of macro-autophagy which is responsible for mitochondrial degradation. Although the molecular basis for the relationship between mitochondrial dynamics and mitophagy is still poorly understood, multiple studies have demonstrated that alterations in mitochondrial fission/fusion affects mitophagy: for example, studies have demonstrated that mitochondrial fission facilitates mitophagy (48-50), whereas fusion protects against mitochondrial clearance (51-53).

Increasing lines of evidence suggest that there are multiple mechanisms by which mitochondria are degraded through mitophagy in the heart. The availability of these multiple options may allow cardiomyocytes to survive even when one mechanism fails. Subsarcolemmal mitochondria are located close to the cellular surface and they may thus be subjected to ejection from cardiomyocytes more frequently than perinuclear or intermyofibrillar mitochondria, although this hypothesis remains to be tested (54).

One of the most well-defined mechanisms through which damaged mitochondria are degraded by mitophagy is the PINK1-Parkin–dependent mechanism [for reviews see references (54-56)]. When mitochondria are damaged and depolarized, PINK1, a mitochondrially targeted serine/threonine kinase, is stabilized and recruits Parkin, an E3 ligase, to mitochondria through the phosphorylation of either ubiquitin (57, 58) or Mfn2 (59). PINK1 alone or K63-linked ubiquitin chains that are formed on the outer mitochondrial membrane recruit LC3 receptors or adapters (for example NDP52, optineurin, p62, NBR1, and NIX), thereby promoting autophagosome formation, a process facilitated by PINK1-mediated phosphorylation of ubiquitin and Parkin (60, 61). These mechanisms allow damaged mitochondria to be engulfed by autophagosomes through LC3-dependent mechanisms. Once the damaged mitochondria are sequestered, the autophagosome will fuse with the lysosome where degradation of its cargo will occur via acid hydrolase enzymes (62, 63) leading to recycling and reuse of its resultant components (lipids and amino acids) during mitochondrial biogenesis (for a review see reference 3).

This is not, however, the only mechanism to identify and target defective mitochondria. Bcl-2 adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and its homologue Nix can also act as a sensor of mitochondrial oxidative stress in response to I/R (63–65), with the oxidation of the N-terminal cysteine residue of BNIP3 and its subsequent homodimerization/activation serving as a signal to initiate mitophagy (64, 66). In addition, the generation of ROS can lead to the oxidation, redistribution, and externalization of the mitochondrial lipid, cardiolipin (CL), which also acts as a signal for mitophagy (67-69). BNIP3 and FUNDC1 may also influence mitochondrial dynamics via complex interactions with both Drp1 and OPA1(65, 70): under normal circumstances FUNDC1 participates in the control of mitochondrial fusion through its interaction with OPA1 while, in the setting of hypoxia, dephosphorylation of FUNDC1 induces dissociation from OPA1 and its subsequent association with Drp1 to promote fission (71).

Myocardial I/R has been associated with activation or upregulation of mitophagy pathways (72–75). In view of its ability to clear depolarizedly damaged mitochondria resulting from I/R, mitophagy can be considered as a protective or adaptive mechanism. However, whether this is indeed the case, or whether it exacerbates cell death, is still a matter of debate, since uncontrolled or excessive (maladaptive) mitophagy may result in the shortage of functional or healthy mitochondria for ATP generation, leading to compromised cell survival. Further investigation is required to clarify the underlying molecular mechanism of mitophagy and its specific role particularly during myocardial reperfusion.

Importantly, increasing evidence suggests that mitochondria can also be degraded by other pathways, which includes Parkin-independent mechanisms of autophagy (49) and autophagy-related Atg5- and Atg7-independent, noncanonical autophagy (76, 77). Sadoshima and co-workers (78) recently showed that mitophagy plays an essential role in preventing mitochondrial and cardiac dysfunction during pressure overload in the heart *in vivo*. In this study it was found that mitophagy was activated after the canonical form of autophagy was inactivated, suggesting that mitophagy in the heart may be mediated by a mechanism distinct from the canonical mechanisms of autophagy (78).

Using suitable mice models subjected to coronary artery ligation *in vivo* and primary cultures of neonatal rat ventricular cardiomyocytes subjected to hypoxia, Sadoshima and coworkers (77) suggested an alternative autophagy process to be the predominant form of mitophagy during myocardial energy stress. It is well-established that adenosine monophosphate-activated protein kinase (AMPK), the conserved sensor of intracellular energy, is activated in response to myocardial stress. AMPK, in turn, phosphorylates Ulk1 at multiple serine/threonine residues, including S555 (79). The subsequent interaction of Ulk1 with Rab9 is dependent on the phosphorylation of S555. Ulk1 directly phosphorylates Rab9 at S179 and recruits a fission complex consisting of Ulk1, Rab9, Rip1 and Drp1 to the damaged mitochondria. Drp1 is phosphorylated at S616 by Rip1, resulting in its activation and mitochondrial fragmentation. Rab9-mediated autophagosomes subsequently engulf the fragmented mitochondria. These events have been illustrated in Figure 1.

In summary, Sadoshima and his coworkers (77) presented compelling evidence that mitophagy mediated by the Ulk1/Rab9/Rip1/Drp1 pathway predominantly mediates mitophagy and is chiefly responsible for the maintenance of mitochondrial quality in the heart during stress. It was suggested that this large protein complex may contain the machinery that forms Rab9-containing autophagosomes, as well as the fission complex, in close proximity to damaged mitochondria (54). The identity of the proteins in the large protein complex and how Rab9-positive autophagosomes are formed and mitophagy is executed remain to be clarified. Moreover, as far as we are aware, the effects of reoxygenation /reperfusion on this pathway have not been evaluated. Interestingly, a recent study from Sadoshima's group demonstrated that the alternative pathway also protects the heart against obesity-associated cardiomyopathy (54), which may be important in the beneficial actions of melatonin in diabetes (see later).

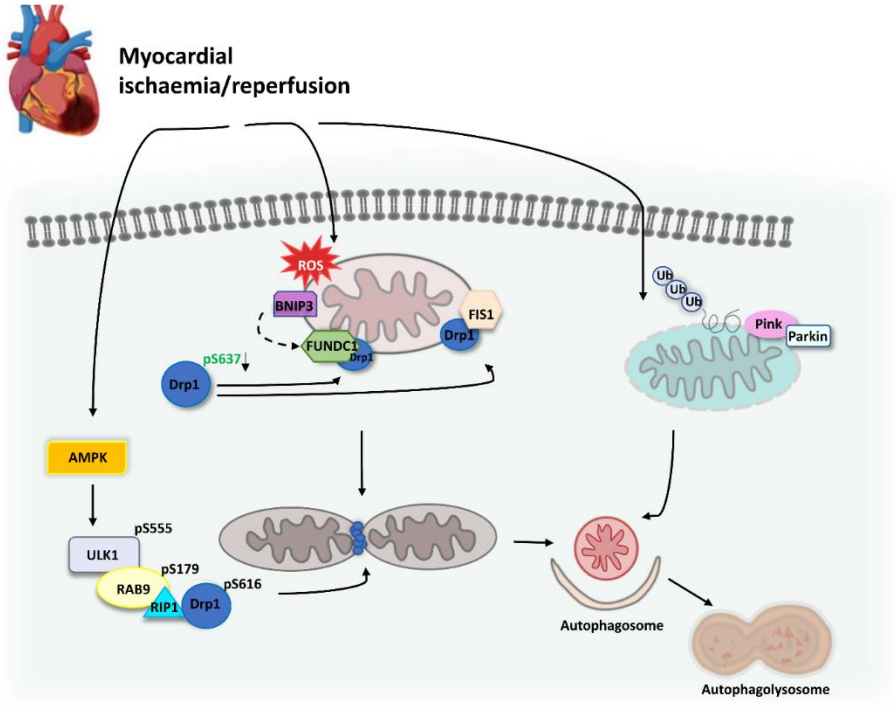

Fig. 1. Fission and mitophagy in ischemia/reperfusion injury.

I/R causes depolarization of mitochondria (shown in dashed lines on the mitochondrion right) and degradation of damaged mitochondria through the PINK1-Parkin-dependent mechanism. PINK1 is stabilized on the outer membrane and recruits Parkin through phosphorylation of ubiquitin (Ub) or Mfn2 (not shown), which leads to the sequestering of, and engulfment by, autophagosomes that fuse with the lysosomes to form autophagolysosomes. Alternatively, Drp1 translocates from the cytosol to the outer mitochondrial membrane under certain conditions. Drp1 oligomerization leads to mitochondrial fission via its interaction with outer membrane proteins, including fission factor 1 (FIS1), whereas Drp1^{pSer637} dephosphorylation during reperfusion induces its translocation from the cytosol to the mitochondria and increases mitochondrial fission. BNIP3, a mitochondrial oxidative stress sensor, and FUNDC1 may also influence mitochondrial dynamics via complex interactions with both Drp1 and OPA (shown as a dashed arrow): in the setting of hypoxia, FUNDC1 dephosphorylation induces dissociation from OPA1 and its subsequent association with Drp1 to promote fission. Lastly, myocardial stress activates AMPK, which in turn phosphorylates Ulk1 at multiple serine/threonine residues, including S555. The subsequent interaction of Ulk1 with Rab9 is dependent on the phosphorylation of S555. Ulk1 directly phosphorylates Rab9 at S179 and recruits a fission complex consisting of Ulk1, Rab9, Rip1 and Drp1 to damaged mitochondria. Drp1 is phosphorylated at S616 by Rip1, resulting in its activation and mitochondrial fragmentation. Rab9-mediated autophagosomes subsequently engulf the fragmented mitochondria.

5. MELATONIN AND MITOCHONDRIA

As mentioned in the introduction, restoration of blood flow after a period of ischemia, aggravates tissue damage due to many pathophysiological mechanisms, particularly the increased generation of ROS and RNS species. There has been an active interest in the development of novel adjunctive therapeutic strategies aimed at protection against oxidative stress, to limit myocardial I/R injury. Amongst these, melatonin, a molecule secreted mainly by the pineal gland, has received much attention. It is an amphiphilic molecule which facilitates its crossing through physiologic barriers binding to specific receptors as well as to several cytosolic proteins, activating a vast array of signaling pathways (for a reviews see references 80-83).

An intricate relationship exists between melatonin and its actions and the mitochondrion *per se* [for reviews see Reiter *et al*. (84,85)]. It is now known that mitochondria can take up melatonin, a process mediated by the oligopeptide transporters GLUT transporter/solute carrier family 2A (GLUT/SLC2A10) and PEPT1/2 (SLC15A1/2) (86, 87). A recent study (88) in mouse brains showed that melatonin is also synthesized in the mitochondrial matrix due to the presence of two key melatonin biosynthetic enzymes, namely arylalkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT). Interestingly, melatonin has been reported to be present in high concentrations in the mitochondria (89-91), the site where free radicals are produced in abundance (92,93).

Melatonin is particularly known for its capacity to scavenge ROS and RNS (94-97). In addition, its metabolites, for example AFMK (N^1-acetyl-N^2-formyl-5- methoxykynuramine) and AMK (N^1-acetyl-5-methoxykynuramine) also scavenge free radicals (98). It also induces the production of endogenous antioxidants: melatonin has been demonstrated to stimulate the activities of superoxide dismutase (99), catalase and glutathione peroxidase(100). Urata and coworkers (101) showed that melatonin increased glutathione levels by stimulating its rate-limiting enzyme, gamma-glutamylcysteine synthase. The versatile and collective protection exerted by melatonin

against oxidative stress may be viewed as a result of the combined antioxidant effects of the parent molecule and its metabolites [for a review see Galano & Reiter (102)]. Therefore, melatonin is classified as a specific mitochondria-targeted antioxidant, acting against the free radicals formed during the electron transport processes (103).

A large number of recent studies defined the critical role of melatonin in maintaining not only the optimal physiology of the mitochondria, but also its ability to protect molecules in the inner mitochondrial membrane against the toxic effects of ROS (104-106). Thus, the beneficial actions of melatonin at the level of the mitochondrion can be summarized as the ability to quench free radicals, reducing oxidative stress, thereby limiting mitochondria-related apoptosis, and maintaining the efficiency of respiratory complexes (107, 108). As expected, loss of melatonin has serious consequences, for example enhanced oxidative stress and diminished ATP production accompanied by elevated ROS generation. [for reviews see references (105, 109)].

The ability of melatonin to protect the heart against I/R damage is well-established and has been reviewed extensively [see for example references 98, 110-112) and as recently as 2022 by Pourhanifeh (113), Tobeiha (114) and co-workers. A systematic review and meta-analysis of the effects of melatonin on the cardiovascular system in experimental models of myocardial infarction have recently been published (115). The main results of this study confirm that melatonin benefits the cardiovascular system suggesting that melatonin could be a useful therapeutic target to combat various cardiovascular diseases. Interestingly, melatonin has been shown to protect against I/R injury in various other organs as well, including the liver (116, 117), brain (118), kidney (119), intestine (120), lung (121) and testes (122).

Using isolated perfused hearts as experimental models, melatonin pretreatment of experimental animals (administered by intraperitoneal injection or added to drinking water) or addition of melatonin directly to the perfusion medium reduced infarct size after coronary artery ligation (123–126), reduced the incidence of reperfusion arrhythmias (127) and improved functional recovery during reperfusion (128–132). The significant antioxidant and anti-apoptotic actions of melatonin have been reported repeatedly in experimental animals (*in vivo* and *in vitro*) and H9c2 cells [for a summary see Pourhanifeh, reference (113), Table 1; Tobeiha, reference (114); Table 3]. Recent *in vitro* and *in vivo* studies using rats showed that melatonin inhibited autophagy (132-134), although it is still controversial whether this effect is positive or negative. The beneficial actions of melatonin on the outcome of I/R have been attributed to its ability to keep the MPTP in the closed confirmation: mitochondria isolated from melatonin-treated reperfused hearts are less sensitive than mitochondria from untreated reperfused hearts to MPTP opening as demonstrated by their higher resistance to Ca^{2+} (135, 136). In addition, melatonin limits ROS production, prevents loss of mitochondrial complex I and III activities, state 3 respiration, release of mitochondrial NAD^{+} and cytochrome c and the mitochondrial cardiolipin (CL) oxidation. Cardiolipin, a phospholipid localized almost exclusively in the inner mitochondrial membrane, is particularly rich in the unsaturated fatty acid linoleic acid and plays a pivotal role in mitochondrial bioenergetics, dynamics and quality control through fusion and fission (136–139). The protective effect of melatonin against CL oxidation can be explained by the ability of the indole to prevent peroxidation of linoleic fatty acids (136).

These observations become even more meaningful if the great deal of variation in experimental conditions are considered. The variation in melatonin concentration in *in vitro* studies and melatonin dosage in *in vivo* studies, as well as the mode of administration (orally, intraperitoneally, added to the incubation medium or perfusate), the time and duration of administration (before

ischemia or during reperfusion) and endpoints, to name but a few, did not affect the beneficial effects of melatonin [see Pourhanifeh (113), table 1].

As was the case in studies on the cardioprotective actions of melatonin, a vast number of experimental models were used in the evaluation of its effects on mitochondrial quality control, ranging from *in vivo* studies in mice and rats to use of primary cardiomyocytes, H9c2 cells, vascular smooth muscle cells (VSMC) and cardiac microvascular endothelial cells (CMEC). Interestingly, while the amounts of melatonin administered intraperitoneally to animals did not vary much (usually 10-30 mg/kg/day (140,141), the amounts of melatonin used in cell studies varied enormously, from 5 μM in cardiomyocytes (142, 143) and VSMC (144) to 10 mM (145) in H9c2 cells. According to Wu and coworkers (146), melatonin at a concentration of 150 μM proved to be most effective in H9c2 cells in culture. However, as stated above, regardless of the model or the dosage, the beneficial actions of melatonin on I/R injury or mitochondrial dynamics were always reported. In several studies observations were made directly in cell cultures (148–151) while in others evaluation of fission and mitophagy was done on mitochondria isolated from hearts subjected to I/R (141,151–155). I/R-induced mitophagy was also studied in mitochondria isolated from cardiac microcirculation endothelial cells (141). As far as we are aware, only one study was done on the temporal effects of melatonin administration during I/R (152): in this study melatonin (10mg/kg via iv injection) was given either(1) 15min prior to cardiac ischemia (2) or15 min after left anterior descending coronary artery ligation (i.e. during ischemia or (3), respectively, at the onset of reperfusion. Treatment with melatonin at all time points alleviated cardiac injury to the same extent, as indicated by, amongst others, the reduction in infarct size and an improvement in mitochondrial function and dynamics.

The effects of this compound on mitochondrial dynamics in the heart and their significance in cardioprotection are currently being evaluated by several groups and will be discussed below. Particular attention will also be given to the effects of melatonin on the quality control of mitochondria isolated from diabetic hearts.

5.1. Mitochondrial oxidative phosphorylation function.

The deleterious effects of I/R on the mitochondrial oxidative phosphorylation process are well-established (5-7, 156–160). It was demonstrated several years ago that melatonin treatment of isolated hearts protects mitochondrial function against I/R injury (158, 161, 162): it was shown that melatonin improves the oxygen uptake, complex I and complex III activity, and increases the CL content of mitochondria prepared after reperfusion of isolated hearts (158, 161). Much of the research done in this regard comes from the laboratory of Petrosillo and Paradies (136, 158, 160) who showed that a loss of CL, due to ROS-induced lipid peroxidation, was responsible for the loss of mitochondrial respiratory chain complexes (Complex I, III IV).

5.2. Mitochondrial dynamics.

Data obtained in multiple models of I/R have shown that restoration of oxygen to cardiomyocytes is associated with a very rapid (< 5min) translocation of Drp1 from the cytosol to the mitochondria (14, 42, 163-166). Compared to controls, I/R induced by coronary ligation in small animals, as well as hypoxia/reoxygenation (H/R) of cardiomyocytes or H9c2 cells, are characterized by increased expression of Drp1, MFF and Fis (128, 133, 138). Simultaneously, the expression of OPA1, Mfn1 and Mfn2 is reduced (142, 145, 147, 152, 165), indicating significant

reduced fusion under these conditions. The causal factors for these changes have been thoroughly reviewed by Hernandez-Resendiz (37), Kulek (4), Anzell and coworkers (55).

5.2.1. Fusion.

An in depth study on the effects of melatonin on OPA1 manipulation was done by Zhang and coworkers (142) using coronary artery ligation *in vivo* as well as H/R of cardiomyocytes isolated from WT mice and $OPA1^{CKO}$ mice. Their results indicated that OPA1 expression, mitochondrial fusion, and mitophagy were significantly depressed by I/R injury, accompanied by infarction area expansion, heart dysfunction, myocardial inflammation and cardiomyocyte oxidative stress. The study provided evidence that melatonin treatment reduced I/R stress by activating OPA1 related mitochondrial fusion, which in turn was dependent on the AMPK signaling pathway, since the beneficial effects of melatonin could be abolished by treatment with compound C, an inhibitor of AMPK (146, 149). Thus, melatonin treatment maintained myocardial function and cardiomyocyte viability, and these effects were highly dependent on OPA1-related mitochondrial fusion/mitophagy, as confirmed by the finding that the beneficial effects of melatonin were reversed by OPA1 ablation. At the molecular level, OPA1-related mitochondrial fusion/mitophagy, which was normalized by melatonin, substantially rectified the excessive mitochondrial fission, promoted mitochondrial energy metabolism and sustained mitochondrial function. Several other groups reported a similar reduction in OPA1, Mfn1 and Mfn2 expression in H9c2 cells exposed to H/R (140, 145, 152), which could be reversed by melatonin. Using similar experimental models, Ma and Dong (147) also reported that I/R reduced expression of fusion factors (OPA1, Mfn1, Mfn2) and increased expression of fission factors (Drp1, MFF) which were reversed by melatonin treatment. OPA1 knockdown abrogated the melatonin effects and induced mitochondrial damage. These authors provided evidence for the first time for a regulatory role of the Yap-Hippo pathway in melatonin actions: I/R reduced Yap expression, which was restored by melatonin. This was further supported by the finding that Verteporfin (a Yap antagonist) inhibited melatonin-induced OPA1 activation. Additional studies are required to determine whether the various Yap subunits have different effects on I/R injury. In summary, the above studies provided evidence for the mechanisms of melatonin-induced reduction of I/R stress by activating OPA1 related mitochondrial fusion, which is dependent on the AMPK signaling pathway.

5.2.2. Fission.

The effects of melatonin on mitochondrial fission have been studied by several groups using different models of I/R or H/R. The involvement of Drp1 in promoting mitochondrial fission is critically dependent on its interaction with CL although the mechanism whereby CL functions in fission, is still unclear (137). It was hypothesized that CL alterations that occur during myocardial I/R (136, 159) may affect mitochondrial fission and dynamics and thereby contributing to mitochondrial dysfunction. Increased mitochondrial fission results in an enhanced susceptibility to mPTP opening, which leads to the release of cytochrome *c* and other caspase family proteins, causing activation of apoptotic cell death at the time of myocardial reperfusion (167).

Without exception and regardless of the variation in melatonin dosage and experimental protocol, it has been invariably reported that the indole was able to reverse the increase in fission induced by I/R (124, 142, 144, 152, 165). In a recent study from our laboratory (124) the effects a

low (0.3 mM) and a high (50 mM) concentration of melatonin on mitochondrial fusion and fission were evaluated in both cytosolic and mitochondrial fractions, prepared at different time intervals of an I/R perfusion protocol of the isolated rat heart. Since melatonin at both concentrations were found to be equally cardioprotective, all subsequent analyses were done on hearts treated with 0.3 mM melatonin. Interestingly, melatonin not only affected the expression of the proteins, but also their phosphorylation and distribution in the cells. Melatonin was found to upregulate cytosolic total (t) and phosphorylated (p) ULK1, throughout the experimental protocol, probably due to AMPK activation. Phosphorylation of ULK1 by AMPK is known to link energy sensing to mitophagy (79). Cytosolic Rab9 levels were also increased by melatonin, particularly during stabilization and reperfusion. Upregulation of the phosphorylation status of serine 616 and serine 637 was used as indicators of activation and inactivation of Drp1 respectively. Melatonin treatment profoundly affected intracellular distribution and phosphorylation of Drp1 causing significant upregulation of cytosolic Drp1^{S637} phosphorylation and the p/t-Drp1 ratio throughout the perfusion protocol. Since upregulation of Drp1^{S637} phosphorylation is indicative of a reduction in its activation and inhibition of translocation to the mitochondria, this indicated that melatonin administration caused a significant reduction in mitochondrial fission during ischemia and reperfusion. These changes occurred concomitantly with a reduction in mitochondrial t-Drp1 expression which may be due to decreased translocation of Drp1 from the cytosol to the mitochondria. However; despite the reduction in mitochondrial t-Drp1, lowering of its phosphorylation and the p/t-Drp1 ratio (particularly during reperfusion), indicated activation of residual mitochondrial Drp1 by melatonin and thus some activation of fission. An attempt was made to calculate whether the inactivation of cytosolic Drp1 exceeds the increased Drp1 activation in the mitochondria: activation of the mitochondrial p/t-Drp1 ratio after exposure to ischemia averaged 35%, compared to 254% increase in cytosolic p/t ratio induced by melatonin (indicative of inactivation). The effects of melatonin on mitophagy *per se* are described in section 4.3.2.

Zhou and coworkers (141) studied the effects of melatonin on the myocardial microcirculation in I/R and elucidated the underlying mechanism. Histological analysis showed that cardiac microcirculation endothelial cells (CMEC) in melatonin-treated mice had an unbroken endothelial barrier, increased endothelial nitric oxide synthase expression, unobstructed lumen, reduced inflammatory cell infiltration, and less endothelial damage. The significance of AMPKa in the actions of melatonin was shown by the finding that its deficiency abolished the beneficial effects of melatonin on microvasculature. *In vitro*, I/R activated Drp1-dependent mitochondrial fission, which subsequently induced voltage-dependent anion channel 1 (VDAC1) oligomerization, hexokinase 2 (HK2) liberation, mPTP opening, PINK1/Parkin upregulation, and ultimately mitophagy-mediated CMEC death. However, melatonin improved CMEC survival via activation of AMPKa, followed by p-Drp1^{S616} downregulation and p-Drp1^{S37} upregulation, which blunted Drp1-dependent mitochondrial fission. This study suggested that melatonin also protects cardiac microvasculature against I/R by its inhibitory effects on mitochondrial fission-VDAC1-HK2-mPTP-mitophagy axis via activation of AMPKa.

Interestingly, melatonin also affects vascular calcification. Mitochondrial fission is known to play a role in cardiovascular calcification. The effects of melatonin on vascular calcification were investigated in vascular smooth muscle cells (VSMCs), prepared from rat aortas (144). Calcium deposits were visualized by Alizarin red staining, while calcium content and alkaline phosphatase activity were used to evaluate osteogenic differentiation. Melatonin markedly reduced calcium deposition and alkaline phosphatase activity, mitochondrial superoxide levels and decreased mitochondrial fragmentation. The hormone also significantly activated the expression of AMPK

and decreased Drp1 expression, effects which were ablated by treatment with Compound C. These findings indicated that melatonin protects VSMCs against calcification by inhibiting mitochondrial fission via the AMPK/Drp1 pathway.

5.3. Mitophagy.

5.3.1. Canonical mitophagy.

As expected, myocardial I/R causes excessive mitophagy, as indicated by, amongst others, upregulation of PINK1, Parkin and Beclin 1 expression and increased LC3 II/I ratios (146, 152, 165, 167). Cardiolipin also plays a role in this process: during mitophagy CL interacts with essential role players of mitophagy such as Beclin 1 and recruits the autophagic machinery via its interaction with LC3 (136).

It has been shown that melatonin mitigates this marked upregulation in mitophagy occurring during I/R, using tissues from the infarct area obtained in experimental animals after coronary artery ligation, as well as H9c2 cells subjected to hypoxia/reoxygenation. Melatonin causes a significant reduction in PINK1 and Parkin expression and increases p62 expression (146, 152, 165). Interestingly, the regulatory effects of melatonin on mitophagy occurs in a receptor-independent manner, since both luzindole (a non-specific receptor blocker) and 4-PPDOT (a specific Mt 2 blocker) did not affect the expression of PINK1, Parkin and p62.

In addition to the above, melatonin has been shown to attenuate postmyocardial infarction via its effects on TOM phosphorylation (169). TOM70 participates in mitochondrial quality control by playing a role in the transport of PINK1 into the intermembrane space of the mitochondria (56, 170).

Using cultured hypoxic murine neonatal cardiomyocytes and mice subjected to permanent coronary artery ligation, a significant reduction in TOM 70 mRNA and expression was observed in both models (169). This was associated with increased mitochondrial damage and ROS production as well as a reduction in mitochondrial function. Conversely TOM70 overexpression (achieved by injecting a lentivirus vector expressing TOM70 into the left ventricle) reduced post-myocardial infarct injury. Melatonin pretreatment of isolated perfused heart also resulted in reversing the ischemia-induced reduction in mitochondrial TOM70 expression (124). Interestingly, Pei and coworkers (169) observed that melatonin treatment alleviated mitochondrial impairment, reduced superoxide generation, preserved cardiomyocytes, improved cardiac functional recovery, and reduced fibrosis in control, but not in Tom70-deficient, mice subjected to myocardial infarction suggesting that Tom70 was indispensable to melatonin-induced protection against post-MI injury. Consistent with these results, Tom70 was reported to increase myocardial resistance to an ischemic insult by facilitating importation of Pink1 into mitochondria (170).

In the past decades most studies focused on cardiomyocyte damage in I/R and the role of mitochondria in this regard. However, platelet activation is a major pathophysiological mechanism that underlies I/R injury and recent studies have demonstrated that mitophagy is important for platelet mitochondrial homeostasis by governing their quality and quantity (171). Zhou and coworkers (171) explored the molecular signals for platelet hyperactivity and investigated the beneficial effects of melatonin on platelet reactivity in response to I/R injury. Loss of PPARγ during reperfusion in a mouse model of I/R injury was found to be closely associated with FUNDC1 dephosphorylation and mitophagy activation, leading to increased mitochondrial electron transport chain complex (ETC) activity, enhanced mitochondrial respiratory function, and

elevated ATP production. The improved mitochondrial function strongly contributed to platelet aggregation, eventually resulting in myocardial dysfunction and microvascular structural destruction. However, melatonin significantly suppressed platelet activation via restoration of the PPARγ content in platelets, which subsequently blocked FUNDC1-required mitophagy, mitochondrial energy production, platelet hyperactivity, and reduced cardiac I/R injury. In contrast, genetic ablation of PPARγ in platelets abolished the beneficial effects of melatonin on mitophagy. Thus, manipulation of the PPARγ/FUNDC1/mitophagy pathway by melatonin could be a novel strategy for cardioprotection in the setting of cardiac I/R injury.

5.3.2. Alternative mitophagy pathway.

As discussed above, Sadoshima, Saito and co-workers presented evidence that the Ulk1/Rab9/Rip1//Drp1 pathway predominantly mediates mitophagy during myocardial ischemia, rather than the conventional form of autophagy which is dependent on the autophagy-related 7 (Atg) conjugation system and LC3 (77). As far as we are aware, the effects of melatonin on this pathway have been investigated by one group only (124). Using the isolated perfused rat heart subjected to global ischemia followed by reperfusion, it was shown that melatonin (0.3 mM) profoundly affected the alternative mitophagy pathway demonstrated by (i) increased phosphorylation of cytosolic ULK1 throughout the perfusion protocol (2) a reduction in mitochondrial and an increase in cytosolic Rab9 expression (3) a significant upregulation of cytosolic $Drp1^{S637}$ phosphorylation and the p/t-Drp1 ratio, associated with a reduction in mitochondrial total phosphorylated and p/t-Drp1 ratio. However, the contribution of this pathway to the beneficial effects of melatonin on cardioprotection remains to be demonstrated.

5.4. Melatonin and mitochondrial biogenesis.

Mitochondrial biogenesis regenerates mitochondria and ultimately increases the mitochondrial mass, as evidenced by increased levels of mitochondrial DNA and mitochondria-related proteins. Mitochondrial biogenesis rapidly augments the mitochondrial population in a short time, which is necessary for cell metabolism under stress. Mitochondrial biogenesis was found to be inhibited in a mouse model of cardiac I/R (172, 173).

Although melatonin has been reported to accelerate mitochondrial biogenesis in early porcine embryos (174), differentiating rat dental papilla cells (175) and Alzheimer's disease patients (176), not much is known about its effects on mitochondrial biogenesis in the setting of cardiac I/R injury. Earlier studies focused on the expression of PGC1a as indicator of mitochondrial biogenesis in hearts from type 1 diabetic mice or rats and hyperglycaemic H9c2 cells. For example, the melatonin-induced reduction in mitochondrial Drp1 expression and fission was associated with upregulation of the SIRT1-PGC1a pathway (140), suggesting that melatonin exerts its cardioprotective effect in a SIRT1-PGC1a-dependent manner. Interestingly, 16 weeks of melatonin treatment inhibited the progression of diabetic cardiomyopathy and associated myocardial I/R injury by reducing mitochondrial fission, enhancing mitochondrial biogenesis and mitophagy via re-activation of SIRT6 and AMPK-PGC1a-AKT signaling (151).

Upregulation of AMPK-PGC1a-SIRT3 1 signaling by melatonin was observed in hearts from diabetic rats or mice. Activation of AMPK played a pivotal role in this response since treatment with compound C, an inhibitor of AMPK, inhibited the cardioprotective effects of melatonin (153).

Thus, enhancing mitochondrial biogenesis and reducing mitochondrial oxidative stress were suggested as therapeutic strategies to ameliorate I/R injury in diabetes.

In a recent study Qi and Wang (143) used primary cultures of neonatal mouse ventricular cardiomyocytes to study the effect of low concentrations of melatonin on mitochondrial biogenesis. The mRNA levels of PGC1a, mitochondrial transcription factor A (Tfam) and nuclear factor erythroid 2-related factor 2 (Nrf2) were significantly lower in the anoxia-reoxygenation (A/R) injury group than in the control group. Due to the reduced transcription of these proteins, the protein levels of translocase of inner mitochondrial membrane 23 (Tim23) and translocase of outer mitochondrial membrane 20 (Tom20) were also reduced in cardiomyocytes subjected to H/R injury, suggesting a reduction in mitochondrial mass. Melatonin treatment dose-dependently increased PGC1a, Tfam and Nrf2 levels in H/R-injured cells. Melatonin treatment also restored Tim23 and Tom20 expression, indicating that melatonin can normalize mitochondrial biogenesis during A/R injury. Melatonin treatment also increased the mitochondrial mass and improved mitochondrial function in their model.

It was concluded that melatonin activated the AMPK pathway, which in turn upregulated PGC1a, Tfam, Nrf2 andSirt3 expression which have been reported to increase mitochondrial DNA levels and oxidative phosphorylation-related protein translation.

6. MELATONIN AND MITOCHONDRIA QUALITY CONTROL IN DIABETIC HEARTS

The effects of melatonin treatment on mitochondrial quality control in diabetes have been intensively studied in recent years. It is well-known that insults associated with diabetes (for example oxidative stress and insulin resistance) contribute to mitochondrial dysfunction which plays a significant role in diabetic cardiomyopathy and I/R injury (177, 178). Most of the studies in this regard were performed on mice with type 1 diabetes mellitus, elicited by streptozotocin treatment. H9c2 cells, exposed to a high glucose concentration (33mM) in the incubation medium, simulated the condition in isolated cells. Diabetic mice have a significantly reduced myocardial function, which could be reversed by melatonin treatment (140, 151, 153).

According to Yu and coworkers (153) their study was the first to demonstrate the cardioprotective effect and potential protective mechanisms of melatonin against I/R damage in streptozotocin-induced type 1 diabetes mellitus. AMPK signaling (using compound C) and SIRT3 (using SIRT3 siRNA transfection) were found to be pivotal for melatonin cardioprotection in the diabetic heart as well as H9c2 cells. In a later study these authors also reported activation of SIRT6 signaling by melatonin in hearts from type 1 diabetic rats (151). Involvement of SIRT1 in diabetic cardiomyopathy was indicated by a recent study by Ding and co-workers (140) which showed that melatonin restored the reduced myocardial expression of SIRT1. The significance of SIRT1 in these hearts was demonstrated by the fact that melatonin failed to improve the heart function of SIRT1 knockout mice. Using hyperglycaemic H9c2 cells, overexpression of SIRT1 prevented mitochondrial fission and it was suggested that melatonin reduced Drp1 expression and mitochondrial fission via the SIRT1-PCG1a pathway. Further information regarding the upstream melatonin effects and its interaction with the sirtuins is required.

As was also observed in hearts from control animals, melatonin treatment inhibited mitochondrial fission in type 1 diabetes mellitus (140, 153) without affecting fusion (140), as indicated by the reduced expression of Drp1 and Ser616 phosphorylation and inhibition of translocation to the mitochondria (151).

It is well-established that melatonin caused a significant reduction in mitophagy during reperfusion/reoxygenation of both H9c2 cells (146, 152, 165) and isolated rat hearts (152). In contrast to control hearts, melatonin treatment of STZ-treated rats or mice had the opposite effects on mitophagy: melatonin treatment of STZ-treated rats on a high-fat diet, partially restored mitophagy (FUNDC1 and Parkin) (151). In addition, a dramatic increase in PINK1 and Parkin expression was reported in mitochondria isolated from diabetic hearts treated with melatonin (179). Parkin was found to play an important role in this scenario since Parkin deletion reversed the protective effects of melatonin in this diabetic cardiomyopathy phenotype. Using Parkin $^{-/-}$ and Mst $^{-/-}$ mice, Wang and coworkers (179) demonstrated that melatonin inhibits Mst1 phosphorylation, thus enhancing Parkin-mediated autophagy to eliminate dysfunctional mitochondria and restoring quality control.

In view of its potential clinical significance, further studies are required to unravel the actions of melatonin on the diabetic heart.

7. THE SIRTUINS AND MELATONIN ACTIONS

Melatonin induces activation of two important members of the Sirtuin (silent mating type information 2 homolog) family, namely Sirtuin 1 (SIRT1) and Sirtuin 3 (SIRT3) which, along with PGC 1a, play a key role in the regulation of mitochondrial biogenesis (180). Particular attention has also been focused on SIRT1, SIRT3 as well as SIRT6 in the context of myocardial ischemia/reperfusion and the potential mechanisms of melatonin in modulating their expression and/or activity.(181).

The cardioprotective actions of SIRT1 activation have long been known (182, 183). SIRT1 deacetylates and activates forkhead box O (FOXO), which synthesizes antioxidants, such as manganese superoxide dismutase (MnSOD) and catalase, thereby promoting cellular resistance against oxidative stress (184, 185).

In the heart, SIRT1 negatively regulates proapoptotic proteins Bax and positively regulates expression of the antiapoptotic protein B cell lymphoma-extra large (Bcl-xL) through FOXO activation. SIRT1 expression decreases in the heart after I/R, whereas SIRT1 cardiac-specific overexpression improves functional recovery after I/R via the effects described above (184). Involvement of activation of SIRT1 signaling in the beneficial actions of melatonin, was first described by Yu and coworkers in the type 2 diabetic heart (131). In hearts from normal rats subjected to I/R, the beneficial actions of melatonin could be abolished by inhibition of SIRT1, using EX527, as well as inhibition of the melatonin receptor by the blocker, luzindole (186). Using type 2 diabetic rats, they subsequently showed that the reduction in I/R injury by melatonin was associated with increased SIRT1 expression, which could be abolished by its inhibition (131). Interestingly, in a later study Yu and coworkers (151) reported activation of SIRT6 signaling by melatonin in hearts from type 1 I/R diabetic rats. This was an important observation since the nuclear and mitochondrial sirtuins, SIRT6 and SIRT3 regulate each other's activity (187, 188).
Importantly, SIRT3 activation has also been demonstrated to reduce I/R injury. In fact, SIRT3 has been suggested to be an underlying regulator of the beneficial effects of melatonin (181, 189, 190). However, the potential interaction between melatonin and SIRT3 has not yet been completely elucidated. Recent studies have demonstrated that SIRT3 can promote or restrain autophagy to alleviate I/R injury by stimulating specific downstream targets, including FOXO3a and AMPK (190). Both *in vivo* and *in vitro* data of Yu and co-workers (153) showed that melatonin's beneficial effects on I/R injury could be abolished by Compound C (a specific AMPK signaling blocker).

Using H9C2 cells, they showed that SIRT3 siRNA inhibited the cytoprotective effect of melatonin without affecting the pAMPK/AMPK ratio and PGC1a expression.

Two subsequent reports also confirmed the involvement of melatonin and activation of the SIRT3 signaling pathway in protecting the heart from ROS injury (181, 192). Based on the findings of Zhai *et al*. (181) the role played by SIRT3 was confirmed by the observation that a selective inhibitor of SIRT3 [3-(1H-1,2,3-triazol-4-yl) pyridine, 3-TYP)] negated the effects of melatonin as a cardioprotective agent. A recent *in vitro* study (165) showed that melatonin protected H9c2 cells against simulated ischemia/reperfusion injury: SIRT3-targeted siRNA eliminated the inhibitory effects of melatonin on mitochondrial fission and mitophagy, and reversed the melatonin-induced increase in SOD2 activity. These results indicate that melatonin postconditioning protects H9c2 cells from A/R injury by inhibiting excessive mitophagy and maintaining the balance of mitochondrial fission and fusion in a SIRT3-dependent manner.

In summary the available data clearly shows that SIRT3 is required for melatonin to neutralize oxidative stress at the mitochondrial level. Importantly, the fact that melatonin promotes the deacetylation of mitochondrial SIRT3 and FOXO3a, which results in the stimulation of the antioxidant enzyme, SOD2, leaves unexplained the importance of the direct free radical scavenging component of melatonin in protecting this organelle and the cell as a whole from free radical damage; this issue requires resolution.

A role for SIRT6 has recently been reported in the cardioprotective actions of melatonin in diabetic (streptozotocin-induced) rats fed a high-fat diet (151). Melatonin was found to also activate SIRT6 signaling, the importance of which was demonstrated by the finding that knock-down of SIRT6 or melatonin receptor blockade by luzindole abolished the ameliorative effects of melatonin against necrosis and infarction. Melatonin-induced activation of AMPK-PGC1a- Akt signaling could be reduced by SIRT6 knock down.

The relative significance of SIRT1, 3 and 6 activations in the beneficial effects of melatonin on I/R damage in the above scenario still needs to be established. Unfortunately, none of the studies reported thus far evaluated all three SIRT's in one study.

8. ROLE OF NOTCH 1

Relatively little is known about the role of Notch1 signaling in the myocardial mitochondrial quality control. Yu and co-workers (193) reported that Notch1 and HES (Hairy and enhancer of Split1) expression was robustly upregulated by melatonin treatment of hearts after coronary artery ligation as well as in H9c2 cells exposed to H/R. Melatonin's effects were abolished by either a blocker of the Notch pathway (DAPT) or the melatonin receptor blocker, Luzindole, indicating that Notch1/HES signalling could play a pivotal role in melatonin-induced cardioprotection (193), although no mention was made of mitochondrial participation. However, a role for Notch1 was further confirmed by the findings that (i) Notch deficiency impairs mitochondrial integrity and metabolic function (ii) activation of Notch1 by Jagged (a Notch1 ligand) attenuated I/R injury and (iii) Mfn1 was indispensable for protection by Notch1 (148). In the latter study tissues were obtained from mice hearts subjected to I/R and no studies were done on isolated mitochondria. It has recently been shown that Notch1 modulated the dynamic balance between mitochondrial fusion/fission and mitophagy in myocardial cells exposed to I/R (194). However, the underlying mechanisms of Notch signaling in these processes were not well-understood. In a follow-up study (148) using neonatal cardiomyocytes exposed to I/R, it was shown that Notch signaling has cardioprotective effects by promotion of fusion and inhibition of fission and mitophagy. Their

results suggested that Notch inhibits PINK1/Mfn/Parkin signaling and upregulates Mfn1 expression through the cascade-up effect of NICD (Notch intracellular domain)/Akt/Mfn. On the other hand, it down-regulates Drp1 expression and inhibits mitochondrial fission. Their study further showed that Notch1 can reduce mitochondrial lysis, reduce myocardial infarct size and inhibit ventricular remodelling, important for myocardial protection. This cardioprotection was almost reversed by Mnf1 knockdown or Drp1 overexpression, which indicates that the protective effect of Notch1 signalling was dependent on the Mnf1/Drp1 regulated mitochondrial fusion-fission dynamics. Unfortunately, the effect of melatonin was not investigated in the latter study.

9. EFFECT OF MELATONIN ON INTERCELLULAR MITOCHONDRIAL TRANSFER

The possibility that melatonin promotes cell survival via intercellular transfer of mitochondria is an exciting novel addition to the mechanisms whereby melatonin exerts its beneficial actions on damaged cells. Studies have shown that cell-to-cell mitochondrial transfer plays an essential role in regulating cardiovascular system development and maintaining normal tissue homeostasis under physiological conditions. In pathological conditions, damaged cells transfer dysfunctional mitochondria toward recipient cells to ask for help and take up exogenous functional mitochondria to alleviate injury. Intercellular transfer of mitochondria in the cardiovascular system occurs through several pathways, including tunneling nanotubes (TNTs), extracellular vesicles (EVs), naked mitochondria extrusion, and others [for reviews see references (195-197)].

TNTs are dynamic actin-containing membranous protrusions that connect cells with a small diameter (20-500nm) and length of up to 100μm (197-199). Through TNTs, cells transfer not only mitochondria but also membrane proteins, soluble molecules, and other organelles. Studies carried out in cultured cells, as well as in rat and human heart tissues, recently demonstrated that the number of TNTs formed between cardiomyocytes and fibroblasts increases during ischemia, likely impacting on arrhythmogenesis, fibrosis, and injury resistance, representing an emerging therapeutic tool (198). TNT mediated transfer from bone marrow derived stem cells (MSCs) to H9c2 cells rescued the cardiomyocytes from I/R damage (200), while TNT-mediated mitochondrial transfer from MSCs to cardiomyocytes mediates cardioprotection in doxorubicin-induced injury models (201). In another study Figeac *et al.* (202) demonstrated that stressed mouse cardiomyocytes triggered human adipose MSCs to release soluble factors, related to cardiac protection through TNT formation. Nonetheless, more compelling evidence is required to demonstrate to which extent TNT-driven mitochondrial transfer contributes to the observed phenotypes. The transfer of healthy mitochondria toward injured cells has several possible protective mechanisms including improvement in mitochondrial biogenesis, enhancement of antioxidative capacity and reduction of apoptosis (199). Unfortunately, the study of TNT-mediated communication has been hampered by technical constraints related to their fragile and transitory nature and the lack of specific molecular markers.

As far as we are aware the effects of melatonin treatment on intercellular mitochondrial transport have not been studied in the I/R heart. However, the effects of melatonin on mitochondrial transfer via TNT's were recently investigated in hippocampal HT22 cells subjected to oxygen/glucose deprivation (OGD) followed by reoxygenation (203). As observed in heart tissue, melatonin (50 μM) upregulated mitochondrial fusion and reduced fission in these cells. Their results also showed that the TNT number was low in controls, but higher in OGD/R cells and significantly higher in OGD/R cells treated with melatonin. The number of mitochondria present in the TNT's showed a similar increase upon melatonin treatment. Whether the improved

transfer of mitochondria between cells occurring after melatonin treatment could be due to its upstream protective effect on the mitochondrial network and which signaling pathways are involved need to be investigated. However, this study provided new insights into the effect of melatonin on the reshaping of the mitochondrial network and neuronal survival. It was speculated that should this effect also occur *in vivo* and melatonin could represent a potential tool to improve survival after MSC cell transplantation in different organs. However, this hypothesis needs to be tested.

Yip and co-workers (204) using N2a cells in culture, also reported that melatonin at a concentration of 200 μM rescued cerebral ischemic events via upregulating TNT-mediated mitochondrial transfer leading to an increased number of mitochondria and downregulation of mitochondrial oxidative stress in neuronal cells.

As stated above, the contribution of melatonin-induced intercellular mitochondrial transfer to its significant cardioprotective potential still needs to be established.

10. MELATONIN, MITOCHONDRIAL-ENDOPLASMIC RETICULUM (ER) TETHERING AND CALCIUM

It is well-established that the sarcoplasmic reticulum Ca^{2+}ATPase (SERCA2a), the inositol phosphate receptor (IP3R) and the mitochondrial calcium uniporter (MCU) play major roles in regulating intracellular calcium homeostasis (205, 209). Over the last two decades, specialized structures called mitochondria-ER contact sites (MERCS) have been identified as critical regulators of cellular homeostasis by controlling tethering dynamics and Ca^{2+} transfer between these organelles (208, 209) . IP3R is localized on the surface of endoplasmic reticulum (ER), whereas MCU is expressed on the outer mitochondrial membrane. Under physiological conditions, regulated Ca^{2+} transfer from ER to mitochondria promotes oxidative phosphorylation and sustains mitochondrial bioenergetics by stimulating ATP synthesis (5, 205). Alterations in mitochondrial-ER tethering can lead to mitochondrial Ca2+ overload and redox imbalance (210, 211) which, in turn, could lead to the opening of the mitochondrial permeability transition pore and cell death (4, 5). Still, despite its potential significance for cardioprotection, the role of mitochondria-ER interaction during myocardial postischemic reperfusion damage remains incompletely understood. Li and coworkers (212) recently investigated the effect of melatonin on mitochondria-ER interaction in an *in vitro* model of myocardial reperfusion damage, using H9c2 cells. Their data confirmed the well-known harmful effects of hypoxia/reoxygenation on these cells (increased ROS production, reduced mitochondrial membrane potential, increased opening of the MPTP and reduced cellular ATP content), which could be reversed by inclusion of melatonin in the incubation medium. In addition, significant ER stress was observed in H9c2 cells exposed to H/R (increases in CHOP, ATF6, caspase 12 mRNA activities), changes which were completely abrogated by melatonin, indicating that melatonin also alleviates ER stress in H/R cardiomyocytes. Furthermore, using qPCR assays, the rapid expression of MERCS genes (Fis1, BAP31, MFN2 and IP3R) after exposure to H/R, was abolished by melatonin treatment, indicating that the indole mediates mitochondrial protection by preventing upregulation of ER stress.

It is not yet clear how melatonin protects cardiac function from reperfusion injury by modulating Ca^{2+} homeostasis. An early study by Yeung and coworkers in 2008 (213) showed that melatonin treatment of rats ameliorated Ca^{2+} homeostasis in myocardial I/R injury in chronically hypoxic hearts. Melatonin treatment significantly mitigated the calcium handling in the hypoxic rat hearts by preserving SERCA expression and it was suggested that melatonin is cardioprotective

against hypoxia-induced myocardial injury by improving calcium handling in the SR of cardiomyocytes via an antioxidant mechanism. However, at the time, no information was available regarding the effect of melatonin on mitochondrial Ca^{2+} fluxes.

As discussed above, the IP3R and SERCA2a play important roles in the maintenance of calcium homeostasis in cardiomyocytes (205, 206, 214–216). Previous studies from Zhou's group confirmed that IP3R upregulation, driven by cardiac I/R injury, is responsible for cardiomyocyte viability reduction and myocardial dysfunction (214, 217-219) associated with mitochondrial Ca^{2+} overload, however, the mechanism is unknown. Interestingly, modulation of the IP3R and SERCA2a by melatonin is dependent on activation of ERK1 (214), suggesting a role for the RISK pathway in this scenario.

The role of the MCU in the defective mitochondrial fusion, fission and mitophagy of myocardial I/R injury was described by Guan and coworkers (154) using mice subjected to I/R as well as neonatal cardiomyocytes in culture subjected to hypoxia/reoxygenation. In addition, they studied the role of the calpains which are activated by Ca^{2+} overload in I/R injury as well as that of OPA1. Their data showed that the expression of MCU was significantly upregulated during I/R and that inhibition thereof (using the inhibitor Ru360) was beneficial, maintaining mitochondrial morphology during I/R. MCU also increased the expression and activation of the calpains during I/R stress, which in turn, down-regulated OPA1-mediated mitochondrial fusion and mitophagy. It was suggested that MCU upregulation contributes to I/R injury via calpain/OPA1-mediated fusion/mitophagy inhibition (154). Using H9c2 cells, subjected to hypoxia/reoxygenation, Hu and coworkers (214) showed a marked increase in cellular Ca^{2+} which could be reduced by melatonin at a concentration of 5 μM, via activation of the ERK pathway, by inhibiting the expression of the IP3R and upregulation of SERCA2a expression. Similar results were obtained in their *in vivo* studies. However, the role of the MCU per se was not investigated in this study.

As far as we are aware, the effect of melatonin on the MCU has only been studied in the cardiorenal syndrome type-3 (CRS-3) (205). Renal I/R reduces cardiac diastolic function associated with cardiomyocyte death and inflammatory responses and disrupts cardiomyocyte energy metabolism, induces calcium overload, and impairs mitochondrial function, as evidenced by reduced mitochondrial membrane potential and electron transport and increased mitochondrial fission. Further, renal I/R induces phosphorylation of the myocardial IP3R and increases expression of the MCU, resulting in cytoplasmic calcium overload and mitochondrial calcium accumulation. Pretreatment with melatonin attenuates renal IR-mediated cardiac damage by maintaining myocardial diastolic function and reducing cardiomyocyte death. Melatonin also inhibits cardiomyocyte IP3R phosphorylation and MCU expression, thereby alleviating cytoplasmic and mitochondrial calcium overload. Blockade of IP3R has similar cardioprotective effects, whereas MCU activation abrogates the melatonin-mediated cardioprotection. These results show that the negative effects of renal I/R on myocardial viability and cardiac function are caused by induction of IP3R phosphorylation, MCU upregulation, and calcium overload. In addition, their results showed that melatonin prevents the early loss of cardiac diastolic function by normalizing the IP3R-MCU-$[Ca^{2+}]_i/[Ca^{2+}]_m$ signaling pathway, and indicates that it may serve as an attractive therapeutic drug for cardioprotection in CRS-3.

It is clear from the above that the direct effects of melatonin on the MCU and its effects on mitochondrial Ca^{2+} homeostasis in the heart need to be evaluated, particularly in the setting of myocardial I/R. Confirmatory analyses of mitochondrial Ca^{2+} fluxes in myocardial I/R are required to determine whether melatonin acts via inhibition of IP3R mediated mitochondrial Ca^{2+} overload.

11. MELATONIN PROTECTS AGAINST DRUG-INDUCED CARDIOTOXICITY

Interestingly (but not unexpected), melatonin has been shown to reverse the harmful effects of several interventions on mitochondrial behaviour. For example, a recent study (220) showed that melatonin has significant therapeutic potential in protecting against mechanical trauma (MT) - induced cardiac dysfunction by preventing excessive mitochondrial fission. Adult male Sprague Dawley rats were subjected to 5-minute rotations (200 revolutions at a rate of 40 rpm) to induce a MT model. Melatonin treatment (30mg/kg) reduced Serine 616 phosphorylation of Drp1 and inhibited mitochondrial Drp1 translocation and mitochondrial fission in the hearts of rats subjected to MT, which contributed to the reduction of myocardial injury and the improvement of cardiac function. In this case, melatonin's protective effects were attributed to its role in suppressing plasma TNF-a overproduction, which was responsible for Drp1-mediated mitochondrial fission. Thus, the beneficial effects of melatonin were due to attenuation of TNF-a production *per se* and not to inhibition of Drp1 translocation to mitochondria (in contrast to where inhibition of Drp1 translocation to mitochondria is associated with cardioprotection (38, 124)). This study demonstrated, for the first time, that abnormal mitochondrial dynamics are involved in post-traumatic cardiac dysfunction.

The cumulative and irreversible cardiotoxic effects of doxorubicin, which limits its clinical use, are well established. Using two acute DOX-induced cardiotoxicity models (*in vitro* and *in vivo*), it was shown that melatonin inhibited DOX-induced mitochondrial dysfunction and morphological disorders, apoptosis, and oxidative stress via activation of AMPK and upregulation of PGC1a with its downstream signaling (NRF1, TFAM and UCP2) (149). These effects could be reversed by AMPK siRNA or PGC1a siRNA in H9c2 cells and were also negated by the cotreatment with the AMPK inhibitor Compound C *in vivo*. In was suggested that the AMPK/PGC1a pathway activation may represent a new mechanism for melatonin exerted protection against acute DOX cardiotoxicity through preservation of mitochondrial homeostasis and alleviation of oxidative stress and apoptosis. This hypothesis was confirmed in a recent study (168) on rats co-treated with DOX and melatonin. In this model melatonin exerted cardioprotection against the harmful effects of DOX via reduction of oxidative stress, inflammation, autophagy and improvement of mitochondrial function, dynamics balance and biogenesis.

Isoproterenol-induced myocardial damage has been used by several workers to study the effect of oxidative stress on mitochondrial damage and the mechanism of melatonin cardioprotection. Isoproterenol (ISO), a β-adrenergic agonist, causes gross infarcts in the rat heart (221) and its pathophysiological effects were comparable to those found in humans (222). ISO causes myocardial damage by instigating a cascade reaction that generates a huge amount of superoxide anion radicals, hydrogen peroxide and hydroxyl radicals (223, 224).

Using mitochondria isolated from goat hearts, Mukherjee and co-workers (225) provided evidence that increasing concentrations of ISO decreased mitochondrial succinate dehydrogenase (SDH) activity as well as activities of other Krebs' cycle enzymes and altered the mitochondrial redox potential, as well as changing the activity of antioxidant enzymes. Co-incubation of these mitochondria with melatonin ameliorated these changes and it was suggested that this was due to the antioxidant abilities of the indole. In another study (155) rats were pre-treated with melatonin (10 mg/kg body weight; intraperitoneally) before ISO administration (25 mg/kg body weight subcutaneously) and their effect on rat heart mitochondrial structure and function was studied. In this *in vivo* study melatonin was also shown to reduce ISO induced oxidative stress, by stimulating superoxide dismutase activity and removing the inhibition of Krebs' cycle enzymes. It was also

shown that melatonin activates the SIRT1-PGC1α-SIRT3 signaling pathways after ISO administration, which ultimately induces mitochondrial biogenesis. In addition, these workers proposed another possible mechanism whereby melatonin protects antioxidant enzymes by binding to them and masking their binding sites. Investigation of the binding characteristics between melatonin and ISO using isothermal calorimetric binding studies revealed that melatonin sequesters ISO molecules and prevents it from attacking the binding sites of catalase along with occupying and masking the active sites of the catalase enzyme themselves, thereby preventing any interaction between ISO and catalase.

Rotenone, a natural plant-derived substance with insecticidal and miticidal activity is a specific inhibitor of mitochondrial respiratory chain complex I and induces free radical formation (226), mitochondrial membrane permeability, mitochondrial membrane potential depolarization (227), ATP production deficiency, apoptosis (225) and PINK1/PARKIN-mediated mitophagy (229). A recent study (230) showed that melatonin rescued rotenone-induced impairment of embryo development by reducing ROS production and promoting mitochondrial biogenesis.

Sepsis-induced myocarditis is another scenario where melatonin has profound effects (for a review see reference 114). This condition is present in half of all patients with septic shock and is characterized by significant impairment of the left ventricular (LV) systolic and diastolic function. Melatonin has also been found to have a cardioprotective effect against experimental sepsis-induced myocarditis. Using mice subjected to cecal ligation puncture as a model (231), melatonin pretreatment alleviated the resultant cardiac dysfunction and relieved myocardial inflammation. In addition, melatonin ameliorated the impaired TCA cycle in mitochondria isolated from these septic hearts, restored their function and abolished ROS accumulation. The therapeutic effect of melatonin on this model of sepsis-induced myocarditis was attributed to its anti-oxidative capacity. In another study lipopolysaccharide (LPS) was used to establish a septic cardiomyopathy model (232). LPS significantly reduced mitochondrial membrane potential, increased ROS production and markers of ER stress. Interestingly, B-cell receptor associated protein 31 (BAP31) transcription was inhibited by LPS. BAP31 is known to interact with TOM40 within ER-mitochondrial contact sites to control mitochondrial function (233). Melatonin could dose-dependently improve cell viability via preserving mitochondrial function and reducing ER stress. Melatonin also restored BAP31 expression, an effect dependent on the MAPK-ERK pathway. The significance of this observation in melatonin's effects on mitochondrial function remains to be elucidated. The beneficial effects of melatonin on LPS-induced septic cardiomyopathy involves activation of SIRT1: melatonin upregulates SIRT1 expression in this model, whereas Ex527, a SIRT1 inhibitor, abrogated the cardioprotective effects of melatonin in sepsis (234).

Ripk, a kinase which plays a role in the activation of necroptosis, expression of pro-inflammatory genes and sustained translation (235), may also be important in LPS-induced sepsis: it was found to be elevated in tissue samples and its overexpression inhibited the effects of melatonin, suggesting a novel mechanism linking Ripk3-modified mitochondrial performance and ER function (236).

12. SIGNIFICANCE OF THE MITOCHONDRIAL MELATONERGIC PATHWAY

It is important for future research to clarify the presence of the melatonergic pathway in human cardiomyocytes, including as to whether exogenous melatonin upregulates this pathway in cardiomyocytes, as shown in other cell types (237). As discussed above, the data obtained thus far clearly show the importance of the (SIRT1/AMPK)-PGC1α-SIRT3 in the protective effects of

melatonin in I/R. Data in other cell types show SIRT3 to deacetylate and disinhibit the pyruvate dehydrogenase complex (PDC), thereby increasing the conversion of pyruvate to acetyl-CoA. Increased acetyl-CoA not only enhances ATP production from the TCA cycle and oxidative phosphorylation, but is also a necessary co-substrate for the conversion of serotonin to N-acetylserotonin (NAS) by AANAT. Consequently, the capacity of melatonin to upregulate the mitochondrial melatonergic pathway in cardiomyocytes may be intimately linked to wider optimization of mitochondrial function (238). Such future research would also be required to clarify the influence of influence of different 14-3-3 isoforms in the regulation of the conversion of tryptophan to serotonin (requiring 14-3-3 eta stabilization of tryptophan hydroxylase) and the stabilization of AANAT (requiring 14-3-3 zeta). Factors acting to regulate these 14-3-3 isoforms and acetyl-CoA (including genetic and epigenetic) may therefore influence to ability of exogenous melatonin to afford protection in I/R.

As with many other medical conditions (239), there is a growing interest on the role of the gut microbiome and gut permeability in the pathoetiology and pathophysiology of cardiac I/R (240). It is notable that the many beneficial effects of the gut microbiome-derived short-chain fatty acid, butyrate, are mediated via the upregulation of SIRT3 and mitochondrial function, suggesting that butyrate's many beneficial effects may be intimately linked to the upregulation of acetyl-CoA and the melatonergic pathway (239).

13. FINAL CONCLUSION

It is clear from the literature that melatonin is a pleiotropic molecule with many actions (see Figure 2). The current review discussed the intricate relationship between melatonin and the mitochondrion *per se*, with a particular focus on events during ischemia and reperfusion of the heart. The cardioprotective actions of melatonin, demonstrated *in vivo* as well as *in vitro*, have elicited much interest and eventually led to further insight into the role of mitochondrial dynamics in cellular homeostasis. The ability of melatonin to protect against oxidative stress by scavenging ROS and RNS and inducing the production of endogenous antioxidants form the basis of most of its beneficial actions. Of particular interest during the past few years is the observation of the significant effect of melatonin on mitochondrial quality control in pathophysiological conditions such as I/R of the myocardium. Available evidence strongly suggests that the beneficial effects of melatonin on mitochondrial quality control plays a pivotal role in its potent cardioprotective effects. Other significant observations were the effects of melatonin on intercellular mitochondrial transfer, mitochondrial-endoplasmic reticulum tethering and the cardiac microvasculature during myocardial ischemia/reperfusion, to name but a few.

Of particular interest and potential clinical significance, are the profound effects of melatonin on the diabetic heart: it reverses the significant reduction in function, inhibits the progression of diabetic cardiomyopathy which is associated with a significant effect on mitochondrial quality control, as evidenced by a reduction in fission.

Due to the marked cardioprotection observed in experimental *in vivo* and *in vitro* studies, several ongoing clinical studies are aimed at translating from proof-of -concept to therapeutic use. A recent review (241) concluded that the pre-clinical proof-of-concept and early clinical studies (phase 2A) suggest a cardioprotective effect of melatonin in various heart diseases. However, larger phase 3 randomized interventional studies are required to establish melatonin and its agonists as cardioprotective therapeutic agents in humans.

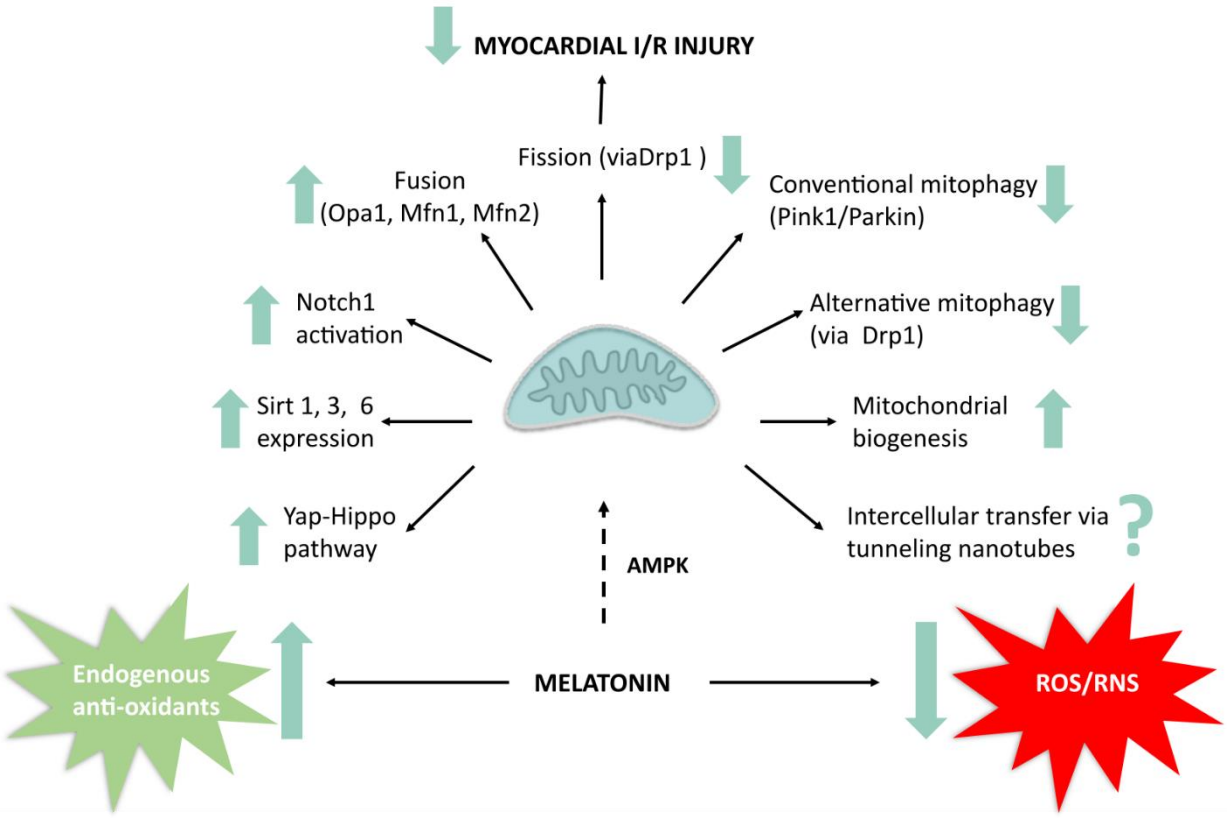

Fig. 2. Summary of the effects of melatonin on mitochondrial behaviour.

Melatonin activates mitochondrial fusion, biogenesis, Notch signaling, SIRT1,3,6 expression and the Yap-Hippo pathway and attenuates mitochondrial fission, conventional and alternative mitophagy.

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AUTHORSHIP

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CONFLICT OF INTEREST STATEMENT

None declared.

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