Review

Molecular mechanisms of melatonin's protection against high-LET radiation: implications for space travel

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ABSTRACT

During a deep space mission, the central nervous system (CNS) and other organs are exposed to galactic cosmic rays and solar particle events. Health risks associated with various organs and systems are important issues in a long-term spaceflight. Potential CNS damage during a space mission could alter cognitive functions which might impact performance and individual's health. The neuronal injury originating from exposure to ^{56}Fe particle irradiation involves the elevated oxidative stress which can be inhibited by melatonin pretreatment. Melatonin exerts potent neuroprotective effects against carbon ion-induced mitochondrial dysfunction and apoptosis in the mouse brain. A significant increase in the count of immature neurons and proliferating cells was detected in the mice under ^{56}Fe particle irradiation cotreated with the melatonin metabolite, AFMK. Melatonin treatment also significantly reduced the carbon ion-induced apoptotic cells and elevated oxidative stress in the mouse testis. The results suggest that melatonin treatment is a potential strategy to protect against space radiation hazards. Spaceflight-induced molecular, cellular, and physiologic changes lead to alterations across many organs and systems. Epigenetic, gene expression, inflammatory, and metabolic responses to spaceflight should be examined and means to safe-guard against these changes in upcoming missions. Precision medicine will be crucial for assessing and augmenting efficacy of melatonin or other medications in astronauts. In addition, enhancing radio-resistance of humans is a novel strategy for a long-term space mission. Further investigations with a combination of melatonin and other novel technologies are warranted to better alleviate HZE particle irradiation-induced damage to astronauts on long-term space exploration missions.

Key words: Melatonin, space exploration, galactic cosmic radiation, solar particle events, high-LET radiation, radio-resistance, CNS.

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1. INTRODUCTION

In recent years, technological advances and space missions have reached the point where the general public is considering traveling to outer space and voyages to Mars may become a reality in the near future. Nevertheless, one of the major problems with space travel is the exposure to galactic cosmic rays (GCR) which consists of high-energy protons and high charge (Z) and energy (E) (HZE) nuclei and solar particle events (SPE) which contains numerous low

to medium energy protons (1). The GCR spectrum consists of about 1% electrons, 85% to 90% protons, 10% to 13% helium ions, and about 1% HZE particles (2), while GCR energy has a wide range from 0.001 to 10^{14} GeV/n, with a peak at 0.1 to 1 GeV/n (3, 4). GCR nuclei are high-LET particles, holding sufficient energies to penetrate any shielding technology employed on contemporary space vehicles (5). The SPE generally consists of 92% protons with energy ranging from 0.01 to 10 GeV/n, 6% helium nuclei and less than 2% HZE particles with energy of several Mev (3). The intense ionization power of GCR ions and SPE containing high fluence and substantial amounts of energetic protons can cause a detrimental biological hazard to astronauts in spacecraft (6). The composition and energy range of GCR and SPE are listed in Table 1.

Table 1. The composition and energy range of galactic cosmic rays and solar particle events.

Type of radiation	HZE particles	Protons	Helium ions	Electrons	Energy range
GCR	1 %	85-90 %	10-13 %	1 %	0.01-10^{14} Gev/n Peak 0.1-1 Gev/n
SPE	2 %	92 %	6%	-	0.01-10 Gev/n

GCR: *galactic cosmic rays*, *SPE*: *solar particle events*, *HZE particles: high charge and energy particles.*

In addition, ionizing radiation causes injury to normal tissues, leading to release of damage-associated molecular patterns (DAMPs), secretion of cytokines and chemokines, and activation of immune system (7). An acute inflammatory phase characterized by an activated pro-inflammatory response and vascular leakage occurs subsequently. A perpetual cytokine and chemokine cascade associated with the recruitment of different immune cells results in many degrees of inflammation (8, 9). T_H1, T_H17 lymphocytes and probably innate lymphoid cells contribute to inflammation (10, 11). A protracted excessive response characterized by activation, proliferation of these immune cells and cytokine secretion is capable of transforming the microenvironment of normal tissues towards the development of severe inflammation (10, 11).

What is needed for these situations is a broadly protective, readily available, easily self-administered, low-cost radiation protector that can be used for a long period of time with little or no serious side effects (12). Melatonin (N-acetyl-5-methoxytryptamine), an indole compound synthesized by the pineal gland and many other tissues is involved in various essential physiological processes (13-16). The protective effect of melatonin against radiation-induced DNA and chromosome damage has been demonstrated in both human (17-20) and experimental animal studies (21-24). Melatonin prevents the death of animals given what would normally be a lethal dose of ionizing radiation (12). Melatonin is an amphiphilic molecule and can easily enter all cells (25, 26). After oral administration, melatonin distributes throughout the body where it prevents radiation-induced damage (10). Melatonin has been safely administered in both physiological and pharmacological doses to humans and animals, and there is a consensus that it is a non-toxic and non-teratogenic molecule (12, 25).

In this review, we summarize the molecular mechanisms of melatonin protection against *high-LET radiation*. In addition, we discuss recent advances related to melatonin as a versatile radioprotector for interplanetary travel by crewed spacecraft.

2. THE EFFECTS OF MELATONIN ON HZE PARTICLE IRRADIATION INDUCED BRAIN DAMAGE

2.1. The effects of melatonin on mitochondrial dysfunction and apoptosis in the mouse brain induced by high-LET carbon ion irradiation.

In the brain, hundreds to thousands of mitochondria are present in a single neuron (27). Mitochondria possess a number of copies of the mitochondrial genome that is composed of a 16.5 kb circular DNA molecule (27). Mitochondria constitute a substantial part of the total cell volume (28). Accordingly, mitochondria are probably important targets of ionizing radiation (28). Ionizing radiation is able to induce numerous lesions in the mitochondrial DNA (29, 30). Ionizing radiation alters mitochondrial functions and induces production of ROS, leading to mitochondrial oxidative stress (28).

Melatonin can serve as the first-line antioxidant to protect against environmental and internal oxidative stress (31). Tan and Reiter summarized the data showing that mitochondria synthesize the potent antioxidant, melatonin (31). The melatonin synthetic enzyme serotonin N-acetyltransferase (SNAT) was immunocytochemically-identified in matrix and also in the intermembrane space of mitochondria almost 40 years ago (31). They also point out that others have identified melatonin membrane receptors, including MT1 and MT2, on mitochondrial membranes (31). The protective effects of melatonin against neuronal injury were proven to be mainly mediated by mitochondrial melatonin receptors rather than the cell surface membrane receptors (31). Melatonin is not only produced in mitochondria but is also metabolized in mitochondria via several pathways, including melatonin's direct interaction with ROS to form a spectrum of metabolites (31).

Various investigations reveal that radiation at doses less than 1 Gy can increase oxidative stress in the brain (32, 33). Overproduction of ROS and reactive nitrogen species (RNS) contributes to destruction of cellular structures and induces apoptosis (32). Apoptosis is recognized as a fundamental process in the nervous system development and disease (34).

A variety of studies reveal that the cellular antioxidant response against ROS is regulated by the nuclear factor erythoid 2-related factor 2 (Nrf2) (35, 36). Nrf2 is a member of NF-E2 family of nuclear basic leucine zipper transcription factors used to eradicate oxidative stressors by binding to the antioxidant responsive element (ARE) or the electrophile-responsive element (EpRE) and is encoded for antioxidant enzymes (36-38).

Liu *et al.* (39) assess the neuroprotective mechanisms of melatonin against carbon ion-induced mitochondrial dysfunction and apoptosis in mouse brain. A significant increase in carbonyl and MDA levels were detected 12 hours after carbon ion irradiation in comparison with the control group, indicating that irradiation promotes the generation of ROS and oxidative stress (39). Melatonin at a concentration of 1, 5 or 10 mg/kg significantly suppressed the carbon ion-induced enhancement of protein carbonyl (PC) and MDA levels in a dose-dependent manner in mice (39). These results highlight melatonin's ability to alleviate the oxidation of protein and lipids following carbon ion irradiation (39).

Superoxide dismutase (SOD) and catalase (CAT) activities and total antioxidant capability (TAC) were significantly suppressed by carbon ion irradiation in comparison with the control group. Melatonin significantly elevated the activities of SOD and CAT, TAC levels as well as the Nrf2 protein expression in a dose-dependent manner in irradiated mice (39).

Apoptotic cells in the mouse brain significantly increased after carbon ion irradiation which can be suppressed by melatonin also in a dose-dependent manner (39).

Carbon ion irradiation reduced Bax is a proapoptotic protein and Bcl-2 is an antiapoptotic protein. The Bax/Bcl-2 ratio is a fundamental determinant of cell death/survival through

apoptosis. This ratio is reduced by carbon ion irradiation; however, melatonin preserves this ratio in a dose-dependent manner (39).

Mice exposed to carbon ion irradiation significantly decreased Rh123 staining, indicating a reduction in ΔΨm (mitochondrial membrane potential). Decreased ΔΨm level is related to mitochondrial dysfunction and subsequent cell death. This alteration also can be prevented by melatonin treatment (39).

Furthermore, carbon ion irradiation induced a significant elevation of cytosolic cytochrome c content, the expression of caspase-3 and its cleavage product. Melatonin at a concentration of 1, 5 or 10 mg/kg suppressed the cytosolic cytochrome c content, the expression of caspase-3 and its cleavage product in irradiated mice (39).

The results reveal that melatonin exerts favorable neuroprotective effects against carbon ion-induced mitochondrial dysfunction and apoptosis in the mouse brain.

2.2. The effects of melatonin on oxidative damage and cell death in the mouse brain induced by high-LET ^{56}Fe particle irradiation.

Manda *et al*. investigated the effects of melatonin on oxidative damage and cell death in the mouse brain induced by high-LET ^{56}Fe particle irradiation (40). Increased necrosis in Purkinje cells and apoptosis of granule cells were detected following ^{56}Fe particle irradiation. Significant reduction in the number of necrotic Purkinje cells and apoptotic granule cells was detected in irradiated mice pretreated with melatonin at a concentration of 10 mg/kg (40).

In different studies, DNA breakage as measured by the comet assay was found in mice following ^{56}Fe exposure. Significant reduction in comet tail length and % DNA in comet tails was achieved by melatonin pretreatment in the irradiated mice. Melatonin also significantly inhibited ^{56}Fe radiation-induced increase in 8-hydroxy-2'-deoxyguanosine (8-OHdG) level, an indicator of oxidative stress (40). Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the products of lipid peroxidation, are extremely harmful to cells (41-42). Melatonin pretreatment significantly inhibited ^{56}Fe radiation-induced elevations of malondialdehyde (MDA) + 4-hydroxyalkenal (4-HAE) and protein carbonyl (PC) level in the cerebellum of mice (40). Pretreatment with melatonin preserves the levels of nonprotein sulfhydryl (NP-SH), an indicator of GSH content and FRAP (ferric reducing antioxidant power of plasma) value, an indicator of total antioxidant capacity (TAC), following^{56}Fe radiation (40).

Thus, brain oxidative damage following high energy charged particle radiation can be efficiently prevented by melatonin pretreatment.

The assessment of the effects of melatonin on charged particle induced brain damage is summarized in Table 2.

Table 2. Assessment of the effects of melatonin on charged particle irradiation induced brain damage.

Parameters	Groups	Results	Reference
8-OHdG level	Control, Mel, IR, Mel + IR	Mel + IR shows a significantly lower 8-OHdG value compared to IR.	40
PC	*Control, IR, Mel (1mg, 5mg, 10 mg/kg) + IR	*Mel + IR shows a significantly lower PC value in a dose-dependent manner compared to IR.	39
	#Control, Mel, IR, Mel + IR	#Mel + IR shows a significantly lower PC value compared to IR.	40

MDA	Control, IR, Mel (1mg, 5mg, 10 mg/kg) + IR	Mel + IR shows a significantly lower MDA value in a dose-dependent manner compared to IR.	39
MDA + HAE	Control, Mel, IR, Mel + IR	Mel + IR shows a significantly lower MDA + HAE value compared to IR.	40
SOD, CAT and TAC	Control, IR, Mel (1mg, 5mg, 10 mg/kg) + IR	Mel + IR shows a significantly higher SOD, CAT and TAC value in a dose-dependent manner compared to IR.	39
NP-SH	Control, Mel, IR, Mel + IR	Mel + IR shows a significantly higher NP-SH value compared to IR.	40
FRAP	Control, Mel, IR, Mel + IR	Mel + IR shows a significantly higher FRAP value compared to IR.	40
Nrf2 expression	Control, IR, Mel (1mg, 5mg, 10 mg/kg) + IR	Mel + IR shows a significantly higher Nrf2 value in a dose-dependent manner compared to IR.	39
Apoptotic cells	*Control, IR, Mel (1mg, 5mg, 10 mg/kg) + IR #Control, Mel, IR, Mel + IR	*Mel + IR shows a significantly lower number of apoptotic cells in a dose-dependent manner compared to IR. #Mel + IR shows a significantly lower number of apoptotic granule cells compared to IR.	39 40
Bax/Bcl-2 ratio	Control, IR, Mel (1mg, 5mg, 10 mg/kg) + IR	Mel + IR shows a significant reduction of Bax/Bcl-2 ratio in a dose-dependent manner compared to IR.	39
ΔΨm	Control, IR, Mel (1mg, 5mg, 10 mg/kg) + IR	Mel + IR shows a significantly higher ΔΨm value in a dose-dependent manner compared to IR.	39
Cytochrome c, caspase-3 and its cleavage	Control, IR, Mel (1mg, 5mg, 10 mg/kg) + IR	Mel + IR shows a significantly lower value of cytochrome c, caspase-3 and its cleavage in a dose-dependent manner compared to IR.	39

8-OHdG: 8-hydroxy-2'-deoxyguanosine, PC: protein carbonyl, MDA: Malondialdehyde, HAE: 4-hydroxyalkenal, SOD: superoxide dismutase, CAT: Catalase, TAC: Total antioxidant capacity, NP-SH: nonprotein sulfhydryl, FRAP: ferric reducing antioxidant power of plasma, Nrf2: the nuclear factor erythoid 2-related factor 2, *ΔΨm: mitochondrial membrane potential, IR: Irradiation only group, Mel: melatonin (10 mg/kg) only group, Mel (1mg, 5mg, 10 mg/kg) + IR: Treatment with melatonin 1 mg/kg, 5 mg/kg and 10 mg/kg before irradiation respectively.*

2.3. The effects of melatonin metabolite, AFMK, on neurogenesis damage induced by high-LET ^{56}Fe particle irradiation in the hippocampal dentate gyrus.

The dentate gyrus of the hippocampus persists in producing new neurons in the adult mammalian brain. Memory functions are related to the pyramidal and granule cells of the dentate gyrus (43). New granule cells are generated from neuronal precursor in the subgranular zone (SGZ). These new cells then move to the granular cell layer (GCL) (43). Neuronal exposure to space radiation may cause a variety of dysfunctional outcomes. Recent evidence indicates that neuronal precursor cells in the hippocampus may be vulnerable to space radiation (44).

N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), a melatonin metabolite, is an infrequently explored biogenic amine. The fundamental principle of kynurenic pathway of AFMK formation is that melatonin interacts with $^1O_2$ and H_2O_2 to generate AFMK, which is converted to N^1-acetyl-5-methoxykynuramine (AMK) by catalase. AFMK is a principal metabolite of melatonin oxidation (43). Manda *et al*. investigated the effect of the melatonin metabolite, AFMK, on neurogenesis damage in the hippocampal dentate gyrus induced by high-LET ^{56}Fe particle irradiation (43).

Immature neurons (Dcx positive) and proliferating Ki-67-positive cells were evaluated in the dentate gyrus. A significant reduction of Dcx positive cells and Ki-67 positive cells was detected in irradiated mice as compared to the control. This reduction is prevented by AFMK at a concentration of 10 mg/kg (43).

Oxidative stress in the brain was usually assessed by levels of malondialdehyde + 4-hydroxyalkenal (MDA + HAE) and protein carbonyl. In brain homogenates of irradiated mice, there was significantly higher levels of MDA + HAE and protein carbonyl as compared to the control. AFMK pretreatment significantly lowers levels of MDA + HAE and protein carbonyl in irradiated mice (43).

Antioxidant status was assessed by TAC level and nonprotein sulfhydryl (NP-SH) content in the brain. There was a significant reduction of TAC level and NP-SH content in irradiated mice and AFMK pretreatment significantly increase of TAC level and NP-SH content (43). The results indicate that AFMK pretreatment exerts neuroprotective effects against ^{56}Fe particle irradiation induced inhibition of neurogenesis in the hippocampal dentate gyrus.

The assessment of the effects of the melatonin metabolite, AFMK, on ^{56}Fe particle irradiation induced neurogenesis damage is summarized in Table 3.

Table 3. Assessment of the effects of the melatonin metabolite, AFMK, on ^{56}Fe particle irradiation induced neurogenesis damage.

Parameters	Groups	Results
Dcx positive cells	Control, AFMK, IR, AFMK + IR	AFMK + IR shows a significantly higher count of Dcx positive cells compared to IR.
Ki-67 positive cells	Control, AFMK, IR, AFMK + IR	AFMK + IR shows a significantly higher count of Ki-67 positive cells compared to IR.
PC	Control, AFMK, IR, AFMK + IR	AFMK + IR shows a significantly lower PC level compared to IR.
MDA + HAE	Control, AFMK, IR, AFMK + IR	AFMK + IR shows a significantly lower MDA + HAE level compared to IR.
NP-SH	Control, AFMK, IR, AFMK + IR	AFMK + IR shows a significantly higher NP-SH value compared to IR.
TAC	Control, AFMK, IR, AFMK + IR	AFMK + IR shows a significantly higher TAC level compared to IR.

Dcx: Doublecortin, AFMK: AFMK (10 mg/kg) only group, AFMK + IR: Treatment with melatonin 10 mg/kg before irradiation.

3. THE EFFECTS OF MELATONIN ON TESTICULAR DAMAGE INDUCED BY CARBON ION IRRADIATION

Space travel poses potential hazards to the reproductive system including testis (45). The testis is one of the most radiosensitive organs in the body (46). Space radiation contains highly

charged and energy (HZE) particles (45). Consequently**,** there is a need for evaluation of the protection of the testis against charged particle radiation.

Liu *et al*. investigated the effects of melatonin on testicular damage induced by high-LET carbon ion irradiation. The comet assay was used to evaluate DNA breakage in mice following carbon ion exposure. Melatonin (10 mg/kg) treatment significantly reduced the carbon ion-induced DNA damage in both groups either melatonin pretreatment or melatonin posttreatment with IR (46).

Melatonin treatment significantly inhibited carbon ion-induced increase in the percentage of apoptotic cells in all of the melatonin plus irradiation groups including melatonin (1 mg/kg) pretreatment +IR, melatonin (10 mg/kg) pretreatment and posttreatment +IR , respectively(46).

Irradiation induced a significant increase in malondialdehyde (MDA) level, and a significant reduction of both glutathione (GSH) and total antioxidant capability (TAC) level and all these alterations are reversed by melatonin treatment (46).

The data support recommendation of melatonin treatment being used to protect against carbon ion-induced testicular damage.

The assessment of the effects of melatonin on carbon ion-induced testicular damage is summarized in Table 4.

Table 4. Assessment of the effects of melatonin on carbon ion-induced testicular damage.

Parameters	Groups	Results
MDA	Control, Mel, IR, Mel pretreatment 1 mg/kg + IR, Mel pretreatment 10 mg/kg + IR, Mel posttreatment 10 mg/kg + IR	Mel + IR shows a significantly lower MDA level compared to IR.
GSH	Control, Mel, IR, Mel pretreatment 1 mg/kg + IR, Mel pretreatment 10 mg/kg + IR, Mel posttreatment 10 mg/kg + IR	Mel + IR shows a significantly higher GSH level compared to IR.
TAC	Control, Mel, IR, Mel pretreatment 1 mg/kg + IR, Mel pretreatment 10 mg/kg + IR, Mel posttreatment 10 mg/kg + IR	Mel + IR shows a significantly higher TAC level compared to IR.
Apoptotic cells (percentage)	Control, Mel, IR, Mel pretreatment 1 mg/kg + IR, Mel pretreatment 10 mg/kg + IR, Mel posttreatment 10 mg/kg + IR	Mel + IR shows a significantly lower percentage compared to IR.

GSH: glutathione, Mel pretreatment 1 mg/kg + IR: Pretreatment with melatonin 1 mg/kg before irradiation, Mel pretreatment 10 mg/kg + IR: Pretreatment with melatonin 10 mg/kg before irradiation, Mel postreatment 10 mg/kg + IR: Treatment with melatonin 10 mg/kg after irradiation.

The results suggest that melatonin treatment is a potential strategy to protect against space radiation hazards.

4. CONCLUSION AND PERSPECTIVE

During interplanetary space travel, the central nervous system (CNS) and other organs are exposed to galactic cosmic rays and solar particle events (1, 47). NASA's radiation standard limits astronaut exposures to a 3% risk of exposure induced death (REID) at the upper 95% confidence interval (CI) of the risk estimate (1). Fatal cancer risk has been considered the dominant risk for space radiation. Risks to the central nervous system (CNS) and other organs are also a concern (1). Potential CNS damage during a space mission could alter cognitive functions which may impact performance and individual's health (48). The neuronal injury

originating from exposure to ^{56}Fe particle irradiation involves damage from elevated oxidative stress which can be prevented by melatonin pretreatment (40). Melatonin exerts potent neuroprotective effects against carbon ion-induced mitochondrial dysfunction and apoptosis in mouse brain (39). A significant increase in the count of immature neurons and proliferating cells was detected in the ^{56}Fe particle irradiated mice pretreated with AFMK at a concentration of 10 mg/kg, indicating that AFMK enhanced neurogenesis in hippocampal dentate gyrus (43). Melatonin treatment also significantly alleviated the carbon ion-induced increase in the percentage of apoptotic cells and elevated oxidative stress in mouse testis. (46). The results suggest that melatonin treatment is a potential strategy to protect against space radiation hazards.

Melatonin meets various criteria as an appropriate radioprotector for the spacefaring population. Firstly, any useful medication should have a history of safe application in humans with minimal or negligible side effects. Secondly, possible side effects of the medication should be easily observed by an astronaut with minimal skill of health care. Thirdly, the medication itself should be easily administered (preferably via the oral route) and should have a long shelf life in the space environment (49). Spaceflight-induced molecular, cellular, and physiologic changes lead to alterations across many organs and systems. Epigenetic, gene expression, inflammatory, and metabolic responses to spaceflight should be examined and means to safe-guard against these changes should be made in upcoming missions. Precision medicine will be crucial for assessing and augmenting efficacy of melatonin or other medications in astronauts (50).

In addition, enhancing radioresistance of humans is a novel strategy for the purpose of interplanetary space travel. Many organisms exhibit high degrees of radioresistance against high doses of radiation (51). Elucidating the genes and molecular mechanisms conferring such high degrees of radioresistance and translating to humans through genetic engineering would constitute an effective strategy to enhance radioresistance in humans (52). *Ramazzottius varieornatus* is one of the most radioresistant species in tardigrades (51). A recent study identified a nuclear protein capable of protecting DNA from the damaging effects of ionizing radiation in *Ramazzottius varieornatus*, termed damage suppressor (Dsup) (53). The human cultured HEK293 cells exposed to 10 Gy of X-ray irradiation revealed that the transfected cells had roughly half as many DNA single-strand breaks as the control group. The neutral comet assay and analysis of the number of γ-H2AX foci (an indicator of double-strand breaks) showed a 40% reduction in DNA fragmentation in the transfected cells compared to the control group. The human cells lost proliferative ability following 3-7 Gy of X-ray irradiation. Following 4 Gy X-ray irradiation the transfected cells had slightly increased viability and higher proliferative ability than the irradiated control cells (53). Therefore, the delivery and expression of Dsup transgenes *in vivo* represent an encouraging candidate for potential exogenous radioprotective transgenes that would assist in establishing enhanced radioresistance in humans for the intention of deep space exploration (52). Mechanistically, the Dsup is a nucleosome-binding protein that protects chromatin from hydroxyl radicals (54). Melatonin accordantly is the most powerful hydroxyl radical scavenger found in all most of the organisms (55). Thus, further investigations with a combination of melatonin and other novel technologies are warranted to better alleviate HZE particle irradiation-induced damage to healthy tissues of astronauts on long-term space exploration missions. The potential mechanisms of melatonin's protection against the high-LET radiation in CNS are illustrated in the Figure 1.

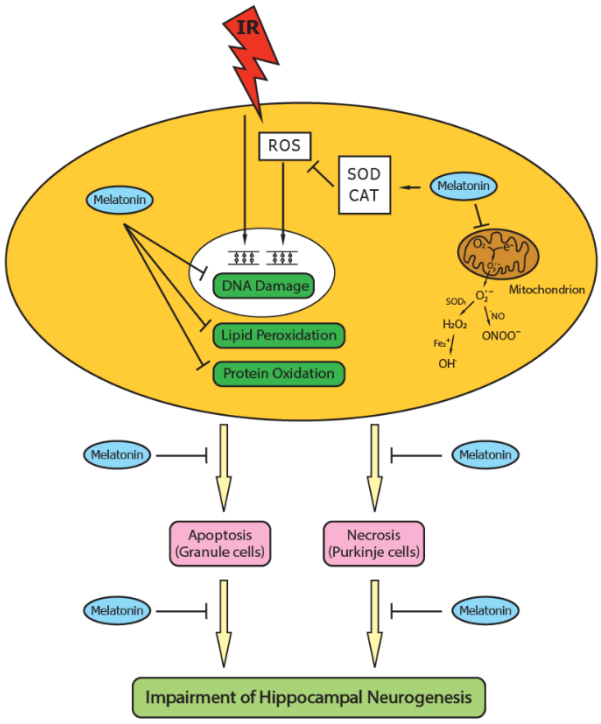

Fig. 1. The potential mechanisms of melatonin's protection against high LET radiation in CNS.

ROS: reactive oxygen species, NO: nitric oxide, SOD: Superoxide dismutase CAT: catalase, arrows: promotion, T type symbol: inhibition.

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AUTHORSHIP

RJR conceived the idea and produced an outline. This was then discussed with the co-author during which time he submitted ideas for inclusion. Both co-authors read preliminary and the final versions of the manuscript.

CONFLICT INTEREST

The authors declare no conflicts of interest.

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