Neuroprotective effect of acute melatonin treatment on hippocampal neurons against irradiation by inhibition of caspase-3

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Abstract. Neuronal cell apoptosis is associated with various factors that induce neurological damage, including radiation exposure. When administered prior to exposure to radiation, a protective agent may prevent cellular and molecular injury. The present study aimed to investigate whether melatonin exerts a neuroprotective effect by inhibiting the caspase cell death pathway. Male Sprague-Dawley rats were administered melatonin (100 mg/kg body weight) 30 min prior to radiation exposure in red light during the evening. In order to elucidate whether melatonin has a neuroprotective role, immunohistochemistry, terminal deoxynucleotidyl transferase dUTP nick-end labeling, Nissl staining, reverse transcription-quantitative polymerase chain reaction, reactive oxygen species analysis and western blotting were performed. At 24 h post-melatonin treatment, caspase-3 mRNA and protein expression levels were significantly decreased. These results demonstrated that melatonin may protect hippocampal neurons via the inhibition of caspase-3 when exposed to irradiation. Therefore, caspase-3 inhibition serves a neuroprotective and antioxidant role in the interventional treatment of melatonin. The results of the present study suggested that melatonin may have a potential therapeutic effect against irradiation; however, further studies are required in order to elucidate the underlying antioxidant mechanisms.

Introduction

Neuronal cell apoptosis is associated with various factors that induce neurological damage, including radiation exposure (1). Free radicals produced by the interaction between ionizing radiation and the biological system attack the components of cells, resulting in cellular damage and apoptosis (2,3). To date, two apoptotic pathways have been extensively characterized, one of which is triggered by the engagement of cell surface death receptors with their specific ligands, and the other is a mitochondrial pathway, triggered by alterations in internal cellular integrity induced by numerous stimuli (4,5). Subsequently, these two pathways induce the activation of caspases (6), which hydrolyze important structural and functional proteins of the cell, ultimately leading to apoptosis (7). Caspases are synthesized in the cell as inactive zymogens and require activation to be functional (7). Radiation induces caspase activation through the mitochondrial pathway, which includes the mitochondrial integration of apoptotic signals and the subsequent release of cytochrome c, Smac and apoptosis-inducing factor into the cytosol (8). This release facilitates the assembly of the apoptosome, which activates caspase-9 and, in turn, leads to the activation of caspases-3, -6 and -7 (9).

Caspase-mediated cell death is associated with the pathogenesis of neuronal degeneration along with other factors such as oxidative damage and inflammation. In addition to activating cell death, previous studies have demonstrated that caspase-3 also has fundamental roles in signal transduction (10,11). Reactive oxygen species (ROS) are byproducts
of the normal cellular metabolism of oxygen; however, a
dramatic increase in ROS levels may lead to oxidative stress. It
has been demonstrated that ROS induced by ionizing radiation
are capable of triggering oxidative cellular damage and stimu-
lating the activation of intracellular signaling pathways (12).
The brain, which is a major metabolizer of oxygen with rela-
tively poor protective antioxidant mechanisms, is particularly
vulnerable to the ROS. In recent years, research in this field
has focused on antioxidant agents that are suitable as radiation
countermeasures (13-15). An effective protective agent against
irradiation administered prior to exposure to radiation may
protect cells from cellular and molecular injury (16).

Melatonin (N-acetyl-5-methoxytryptamine; Mel), which is a
neurohormone and the major product of the pineal
gland, may be a novel therapeutic agent for the treatment of
various disorders associated with inflammation and oxidative
stress (17). It has been reported that Mel was able to improve
short and long-term neurobehavioral deficits and attenuate
hippocampal impairments following hypoxia in neonatal
mice (18). In addition to neutralizing ROS species, Mel also
acts via the stimulation of various anti-oxidative systems and
stabilizes cell membranes (19). As such, Mel modulates the
gene expression levels of numerous protective enzymes
to reduce apoptosis and lipid peroxidation (20,21). Mel has
previously been demonstrated to improve the survival rates
of mice when administered prior to irradiation exposure (22).

The hippocampus is a region of active proliferation and neuro-
genesis within the brain. It has previously been demonstrated
that ionizing radiation induces the apoptosis of neural cells
within the subventricular zone of the lateral ventricles and the
subgranular zone of the hippocampus in the adult brain (23).
Furthermore, previous studies have demonstrated that the
caspase-dependent cytotoxicity of ionizing radiation in hippo-
campal neurons is induced by oxidative stress (24,25).

The present study aimed to investigate whether Mel
inhibits the caspase cell death pathway to protect hippocampal
neurons from irradiation-induced apoptosis. Furthermore,
the underlying mechanisms of this phenomenon within cells
were studied in order to elucidate whether Mel may be a novel
therapeutic agent for the prophylactic treatment of irradiation.

Materials and methods

Animals and reagents. A total of 18 male Sprague-Dawley rats,
weighing 200-220 g and aged 6-8 weeks, were maintained
in individual cages for 1 week at 22±2°C, 40-60% humidity
and under a 12-h light-dark cycle, with ad libitum access to
food and water. All animal experiments were conducted
in accordance with a protocol approved by the Institutional
Animal Care and Use Committee of the Institute of Radiation
Medicine, Chinese Academy of Medical Sciences (Tianjin,
China). Animals were randomly assigned into three groups
(n=6/group): Irradiation (IR) group, irradiation with Mel
(IR + Mel) group and control (Con) group. Mel was purchased
from ImmunoWay Biotechnology Company (Newark, DE,
USA).

Mel administration. Rats in the IR + Mel group were adminis-
tered Mel (100 mg/kg body weight) by intraperitoneal injection;
the IR and Con groups were treated with an equal volume of
isotonic NaCl solution (Fuyu Fine Chemical Co., Ltd., Tianjin,
China) as a vehicle, with and without the proceeding irradiation,
respectively. All treatments were performed 30 min prior
to radiation exposure in red light at 6 p.m.

Irradiation. Rats were placed in ventilated plexiglass
containers (30x25x30 cm; Nanfang Organic Glass Factory,
Tianchang, China) and administered total body irradiation
(TBI) using 137 Cs γ rays (Cammacell-40;Atomic Energy,
Mississauga, ON, Canada) at a dosage of 1.0 Gy/min (26). Rats in
the IR and IR + Mel groups received a total of 4.0 Gy TBI. Rats in the control group were placed in identical containers
for the same period without irradiation.

Tissue preparation. At 24 h post-experimental intervention,
the rats were sacrificed by an overdose with intraperitone-
ally administered sodium pentobarbital (50 mg/kg; Beijing
Biosynthesis Biotechnology Co., Ltd., Beijing, China) and
immediately treated with a cardiac perfusion of 4% para-
formaldehyde (CellChip Biotechnology Co., Ltd., Beijing,
China). The hippocampi were harvested and cut into 12-μm
coronal sections (3 rats/group) using a CM 3000 cryostat
(Leica Microsystems GmbH, Wetzlar, Germany) and were
subsequently placed on glass slides and stored at -80°C (27).

Immunohistology, terminal deoxynucleotidyl transferase
dUTP nick end-labeling (TUNEL) and cresyl violet (CV)
staining. A standard immunohistochemical analysis was
conducted according to a previous study (28). Briefly,
coronal sections were air dried for 15 min, post-fixed in 10% formalin (Hangzhou Norming Biological Technology Co.,
Ltd., Hangzhou, China) for 15 min, washed twice in phos-
phate-buffered saline and then processed for immunostaining
with rabbit anti-active caspase-3 polyclonal antibody (1:1,000;
cat. no. ab2302; Abcam, Cambridge, MA, USA). This was
followed by incubation with horseradish peroxidase-conju-
gated goat anti-rabbit IgG (1:3,000; cat. no. ta14003; OriGene
Technologies, Inc., Beijing, China) and then 3,3′-diaminoben-
zidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO,
USA). Subsequently, the sections were visualized under a light microscope (LSM-510; Carl Zeiss AG, Oberkochen,
Germany).

DNA fragmentation was detected using a TUNEL kit
(In Situ Cell Death Detection Kit, POD; Roche Diagnostics,
Indianapolis, IN, USA) according to the manufacturer’s
protocol and as described previously (29). Briefly, sections were
incubated for 90 min at 37°C with TUNEL reaction mixture.
Positive control sections were incubated with 200 U/ml DNase
I (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA)
for 5 min prior to fixation. Negative control sections underwent
the same procedure but terminal deoxynucleotidyl transferase
was omitted from the reaction buffer to evaluate nonspecific
labeling. TUNEL cell counts were performed on brain sections
(n=3) from the hippocampi. TUNEL-positive cells were aver-
gaged from the counts of three adjacent brain sections of a rat.
The sections were visualized using the Eclipse Ti-U inverted
microscope (Nikon Corporation, Tokyo, Japan) with an excita-
tion/emission wavelength of 500/550 nm (green).

CV staining was performed in order to detect the Nissl
body in the neuronal cytoplasm and to identify the basic
neuronal structure of necrotic neurons in the brain and spinal cord. Sections were rinsed in tap and distilled water, and subsequently stained in 0.1% CV solution (CellChip Biotechnology Co., Ltd.) for 3-10 min. Following rinsing in distilled water, the sections were differentiated in 95% ethyl alcohol (Sangon Biotech Co., Ltd., Shanghai, China), dehydrated in 100% alcohol and cleared with xylene (Sangon Biotech Co., Ltd.), prior to mounting with permanent mounting medium (Yantuo Biological Technology Co., Ltd., Shanghai, China). The Nissl body was stained purple-blue (28).

**Western blot analysis.** Western blotting was performed according to a standard procedure as described previously (29). Briefly, the rats were sacrificed at 24 h following irradiation, and the hippocampi (n=3/group) were obtained. Total protein was isolated from the hippocampi using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology Co., Ltd., Shanghai, China), dehydrated in 100% alcohol and cleared with xylene (Sangon Biotech Co., Ltd.), prior to mounting with permanent mounting medium (Yantuo Biological Technology Co., Ltd., Shanghai, China). The Nissl body was stained purple-blue (28).

![Western Blot](image)

**Figure 1.** Representative images of photomicrographs stained with (A) cresyl violet, (B) caspase-3 and (C) TUNEL in the CA3 region of hippocampi. (D) TUNEL-positive neurons in hippocampi. The box indicates the image positioning of immunohistology and TUNEL. Scale bars, 50 µm. *P<0.01 vs. the IR group. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; con, control; IR, irradiation; Mel, melatonin.

![Caspase-3 Activity Assay](image)

**Figure 2.** (A) Caspase-3 activity assay, expressed as fold change relative to the Con group. (B) DCF fluorescence intensity. *P<0.05 and **P<0.01 vs. the IR group. Data are presented as the mean ± standard deviation. Con, control; IR, irradiation; Mel, melatonin; DCF, dichlorofluorescein probe.
to the manufacturer’s protocol. RNA was treated with DNase (TURBO DNA-free™ kit; Thermo Fisher Scientific, Inc.) and 3 µg RNA was used for cDNA synthesis, as previously described (29). Briefly, RNA was reverse transcribed into cDNA using a using an iScript™ Select cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR was performed using the 2X PCR Master Mix (Beyotime Institute of Biotechnology), an ABI PRISM® 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the following primers: Caspase-3, forward 5'-AAT TCA AGG GAC GGT CAT G-3' and reverse 5'-GCT TGT GCG CGT ACA GTT TC-3'; and GAPDH, forward 5'-ATG ACA AAA TGA GCT TG-3' and reverse 5'-CAT ACC AGG CGT ACA GTT TC-3'; and GAPDH, forward 5'-ATG ACA AAA TGA GCT TG-3' and reverse 5'-CAT ACC AGG CGT ACA GTT TC-3'. The PCR cycling conditions were as follows: 95°C for 20 sec, followed by 50 cycles at 95°C for 3 sec and 60°C for 30 sec, and then a final extension step at 72°C for 5 min. The cycle threshold (Cq) values of caspase-3 were normalized to the Cq values of the GAPDH housekeeping gene using the 2^ΔΔCq method (30). All samples were analyzed in triplicate. The results of the first strand cDNA synthesis step were analyzed by RT- minus control were used to verify the results of the first strand cDNA synthesis step.

ROS analysis and caspase-3 activation assay. A 2',7'-dichlorodihydrofluorescein diacetate (DCF) fluorogenic probe (Hangzhou Boda Biological Technology Co., Ltd., Hangzhou, China) was used to assess the production of ROS, as previously described (31). The activities of caspase-3 were analyzed using a fluorogenic caspase assay with Ac-DEVD-AFC (BD Pharmingen, San Diego, CA, USA) as the substrate. The results were expressed as the fold change, compared with the control, according to technique described by Li et al (28).

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Statistical comparison of the results was performed using paired Student’s t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Mel administration attenuates irradiation-induced neuronal damage. The Nissl body was successfully stained purple-blue (Fig. 1A). Microscopic examination of the hippocampal sections stained with CV showed pathological changes in the IR and IR ± Mel groups, as compared with the control group (Fig. 1A). Viable neurons had a deeply stained cytoplasm and lightly stained nucleus. Immunohistochemical labeling of caspase-3 was more intense in the hippocampi of the IR group than in the IR + Mel group; whereas staining was more intense in the IR + Mel group than the control group (Fig. 1B). TUNEL-positive cells were not clearly observed in the CA3 region of the control group, whilst TUNEL-positive staining was observed in the hippocampi of rats in the IR group with condensed chromatin and fragmented DNA (Fig. 1C). The IR + Mel group exhibited a decrease in TUNEL positivity compared with the IR group (Fig. 1C). Upon quantification of viable cells in the hippocampi, the IR and IR + Mel groups exhibited a decrease in the mean number of surviving neurons, as compared with the control group. However, the number of surviving neurons were significantly increased in the IR + Mel group, as compared with the IR group (P<0.01; Fig. 1D). These results suggest that Mel administration attenuates irradiation-induced damage.

Mel administration attenuates caspase-3 expression levels. The expression levels of caspase-3 were detected by western blot and RT-qPCR analyses. In the IR group, caspase-3 expression levels were significantly increased compared with the control group, at the protein (P<0.01; Fig. 3A and B) and mRNA (P<0.01; Fig. 3C) levels. Caspase-3 protein expression levels were significantly decreased in the IR + Mel group compared with the IR group (P<0.01; Fig. 3A and B), as were caspase-3 mRNA expression levels (P<0.05; Fig. 3C). These results suggested that treatment with Mel significantly attenuated the expression levels of caspase-3.
Discussion

In the present study, a modified protocol was used to induce hippocampal neurodegeneration by irradiation in vivo in order to investigate the potential protective mechanism of Mel on the hippocampi via decreased caspase-3 expression and activity levels. Radiation damage to cells is caused by oxidative stress (32). The increased caspase-3 levels detected in the IR group of the present study demonstrated the role of oxidative mechanisms in irradiation-induced tissue injury. Free oxygen radicals are molecules released from macrophages and neutrophils, which are efficient in the early period of inflammation, that target DNA proteins and lips (33). Appropriate antioxidation intervention, via the inhibition or reduction of free radicals, offers protection against radiation-induced damage. Mel is a highly efficient free radical scavenger and a general antioxidant that has previously been demonstrated to protect DNA, lips and proteins (32,34,35). Furthermore, antioxidative enzyme activities have been shown to exhibit circadian rhythms that correspond to Mel rhythmicity and the total antioxidant status (36). Mel has been reported to increase the activity of important antioxidative enzymes at the molecular level, including superoxide dismutase and glutathione peroxidase (37). The present study investigated the antioxidative and immunoenhancing actions of Mel via the inhibition of caspase-3 in vivo.

As previous studies have demonstrated, Mel acts as a stimulant under immunosuppressive conditions or as an anti-inflammatory compound during immune responses, including acute inflammation (38-41). Advantageously, Mel is ubiquitously distributed in all the cellular compartments, and is capable of quickly passing through all the biological membranes (42). The results of a previous study demonstrated that no significant changes were identified in the Mel levels among the IR, IR + Mel and control groups 24 h after treatment (43). These results may be due to the ability of Mel to stimulate antioxidative enzymes which may maintain their enzyme activity levels following the metabolic decomposition of Mel. Therefore, signal transduction and the expression levels of antioxidative enzymes following treatment with radiation and Mel should be investigated in future studies.

In conclusion, the results of the present study demonstrated that Mel may protect hippocampal neurons from apoptosis via the inhibition of caspase-3 following irradiation. A significant decrease in caspase-3 mRNA (P<0.05) and protein (P<0.01) expression levels was detected at 24 h after Mel treatment. Therefore, caspase-3 inhibition may have a neuroprotective and antioxidative role in the interventional treatment of Mel, and these results demonstrate the potential therapeutic effect of Mel against irradiation. Further studies are required in order to elucidate the underlying effects and mechanisms of Mel on irradiation-induced alterations in caspase-3.

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