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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin blunts the mitochondrial/NLRP3 connection and protects against radiation-induced oral mucositis

Abstract: Mucositis is a common and distressing side effect of chemotherapy or radiotherapy that has potentially severe consequences, and no treatment is available. The purpose of this study was to analyze the molecular pathways involved in the development of oral mucositis and to evaluate whether melatonin can prevent this pathology. The tongue of male Wistar rats was subjected to irradiation (X-ray YXLON Y.Tu 320-D03 irradiator; the animals received a dose of 7.5 Gy/day for 5 days). Rats were treated with 45 mg/day melatonin or vehicle for 21 days postirradiation, either by local application into their mouths (melatonin gel) or by subcutaneous injection. A connection between reactive oxygen species, generating mitochondria and the NLRP3 (NLR-related protein 3 nucleotide-binding domain leucine-rich repeat containing receptor-related protein 3) inflammasome, has been reported in mucositis. Here, we show that mitochondrial oxidative stress, bioenergetic impairment and subsequent NLRP3 inflammasome activation are involved in the development of oral mucositis after irradiation and that melatonin synthesized in the rat tongue is depleted after irradiation. The application of melatonin gel restores physiological melatonin levels in the tongue and prevents mucosal disruption and ulcer formation. Melatonin gel protects the mitochondria from radiation damage and blunts the NF-κB/NLRP3 inflammasome signaling activation in the tongue. Our results suggest new molecular pathways involved in radiotherapy-induced mucositis that are inhibited by topical melatonin application, suggesting a potential preventive therapy for mucositis in patients with cancer.

Francisco Ortiz[1], Darío Acuña-Castroviejo[1,2], Carolina Doerrier[1], José C. Dayoub[1], Luis C. López[1,2], Carmen Venegas[1], José A. García[1], Ana López[1], Huayqui Volt[1,2], Marta Luna-Sánchez[1,2] and Germaine Escames[1,2]

[1]Centro de Investigación Biomédica, Parque Tecnológico de Ciencias de la Salud, Universidad de Granada, and RETICEF, Granada, Spain; [2]Departamento de Fisiología, Facultad de Medicina, Universidad de Granada, Granada, Spain

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Address reprint requests to Germaine Escames, Ph.D., Centro de Investigación Biomédica, Parque Tecnológico de Ciencias de la Salud, Avenida del Conocimiento s/n, 18100 Armilla, Granada, Spain.
E-mail: gescames@ugr.es

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Introduction

Mucositis is a particularly severe and common acute side effect of chemotherapy and/or radiotherapy. Mucositis is a debilitating condition that involves deep mucosal ulcerations due to damage to the normal tissue, and no treatment exists [1]. The main mechanism underlying the development of mucositis is considered to be related to the direct cytotoxic effects of chemotherapy or radiotherapy on the basal cells of the epithelium because of their high cell turnover rate. The cytotoxic effects lead to an ulcerative phase with bacterial colonization and related inflammation. Consequences include prolonged hospitalization, need for parenteral nutrition, severe pain, risk of infection, and increased risk of morbidity and mortality. Once established, mucositis is resistant to treatment efforts except for limiting the dose of radiotherapy. These serious side effects are the major cause of therapy discontinuation, hampering the success of tumor treatment, worsening quality of life and outcomes for patients with cancer, and substantially increasing the cost of care per patient [2]. Palifermin is the only agent that appears to modestly affect the severity of oral mucositis in patients receiving bone marrow transplants; however, it is associated with adverse effects, requires intravenous administration, and is expensive [3, 4]. The need is urgent to identify new molecular targets for developing novel therapies against mucositis [5]. Radiotherapy-induced oral mucositis is incompletely understood and depends on multiple pathogenic events including direct cytotoxicity, cell hypoproliferation, apoptosis, abnormal inflammation, oxidative/nitrosative stress, and reactive oxygen species (ROS) [6]. A connection between ROS-generating mitochondria and the NLRP3 inflammasome has been recently reported in studies unrelated to mucositis [7]. Once constituted, NLRP3 activates caspase-1, which in turn activates the NF-κB-dependent pro-inflammatory cytokine IL-1β [8], forming a new inflammatory pathway not yet explored in mucositis.

Melatonin (N-acetyl-5-methoxytryptamine) is a potent-free radical scavenger with antioxidant and anti-inflammatory properties [9, 10]. Melatonin is also a special type of antioxidant because when scavenging free radicals, it is transformed into a series of metabolites that are also free radical scavengers [11, 12]. Moreover, melatonin is taken up by mitochondria, providing in situ protection against oxidative damage [13]. In vivo, melatonin also restores normal mitochondrial function that has been impaired in different inflammatory conditions [13–15]. In rats, the indoleamine has been shown to protect against radiation-induced enteritis [16] and attenuate methotrexate-induced oxidative stress [17]. The mechanism has been attributed to the scavenging of free radicals and attenuation of lipid

membrane peroxidation, neutrophil-induced infiltration, and cytotoxicity [17].

Here, we show that in addition to activation of the NF-κB pathway, radiotherapy-induced mucositis causes severe mitochondrial oxidative damage and dysfunction, leading to NLRP3 activation, IL-1β release, and apoptosis in rats. We also show that topical melatonin application in the rat mouth inhibits the three pathways activated by irradiation, that is, NF-κB, NLRP3, and mitochondrial dysfunction, impeding the development of mucositis. This study reveals new molecular mechanisms of radiotherapy-induced mucositis and identifies the first potential effective treatment for this disease.

Materials and methods

Animal irradiation

Male Wistar rats were used for this study. The rats were randomly assigned into four groups: control (nonirradiated), irradiated + vehicle (gel without melatonin), irradiated + local application of 3% melatonin gel, and irradiated + intraperitoneal injection of melatonin. A group of irradiated rats was also injected with vehicle propilenglicol:saline (10% v/v) to serve as control for intraperitoneal melatonin. No differences were observed between irradiated rats treated with oral or intraperitoneal vehicles. So, only one vehicle group was included in the figures. The total melatonin dose was 45 mg/day for a total of 21 days.

The tongue was irradiated under anesthesia (1 mL equithesin/kg body weight, IP) at 7.5 Gy/day for 5 consecutive days [18] (Fig. 1A). Ionizing radiation was delivered by an X-ray YXLON Y. Tu 320-D03 irradiator (Yxlon, Hamburg, Germany) using a voltage of 207.3 kV, working current of 10.5 mA, focus of irradiation of 5 mm of diameter, 0.25-mm Cu filter system, target distance of 15 cm, irradiation field of 0.78 cm^2, and delivered dosage of 100.2 cGy/min. For irradiation, each animal was placed into a customized rat jig with standardized snout positioning. A lead shield covered the entire animal except for the mouth. The radiation dose to shielded areas was 2.4×10^{-2} cGy/min. This dose rate ensures that the total body dose received by rats during the irradiation treatment is less than 9.0×10^{-3} Gy. After radiation, animals were housed (four or five animals per cage) and maintained in the

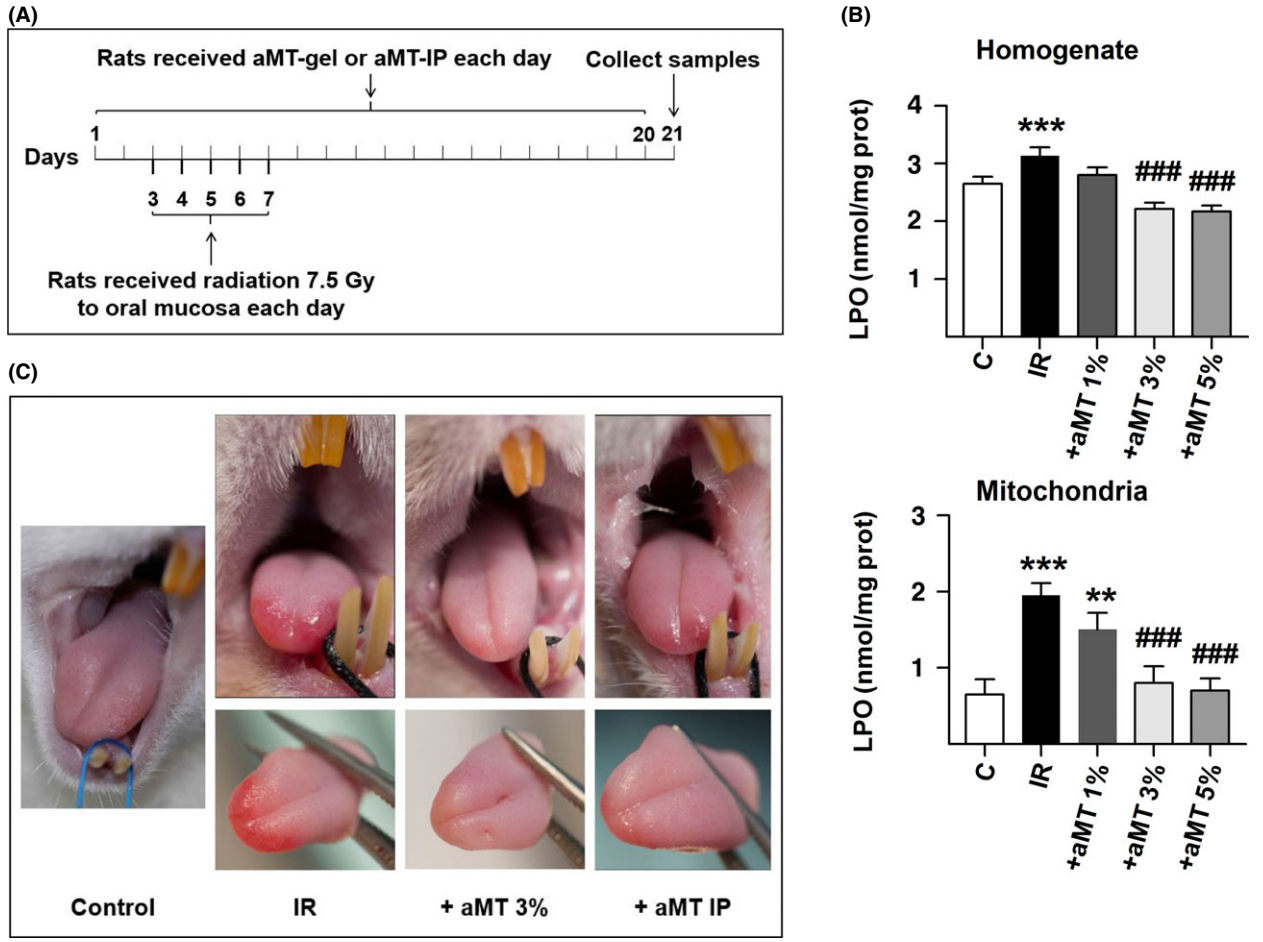

Fig. 1. Melatonin gel 3% counteracted the deleterious effect of fractionated radiation, whereas ip melatonin had a minor effect. (A) Fractionated radiation and melatonin treatment schedule. (B) Lipoperoxide (LPO) levels in homogenate tissue and mitochondria of tongues from control (nonirradiated, C) and irradiated rats treated with vehicle (IR), 1% melatonin gel (+aMT gel 1%), 3% melatonin gel (+aMT gel 3%), or 5% melatonin gel (+aMT gel 5%), $n = 6$ per group. (C) Representative pictures of tongues from control and irradiated rats after they received vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). Data are expressed as mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#\#\#}P < 0.001$ versus IR.

University's facility on 12 hr light:12 hr dark cycle, at 22 ± 2°C with regular chow and water.

Melatonin gel or vehicle was applied in the oral cavity 48 hr before each irradiation dose and up to 14 days after the last irradiation exposure. Melatonin gel was applied three times a day in the intraoral regions using a plastic Pasteur pipette. In the irradiated + ip melatonin (aMT) group, one injection of melatonin was given intraperitoneally each day at the same dose as in the gel (45 mg/day for a total of 21 days). During the irradiation period, the gel was applied 1 hr before and 1 hr after each radiation dose, with the third dose 6 hr later, whereas intraperitoneal melatonin was given 1 hr before the onset of irradiation. Rats were monitored daily and euthanized 14 day after the last dose of radiation. When animals were sacrificed, tongues were harvested and processed appropriately for the different analyses.

Histology and histopathology

Tongue samples were fixed in 10% buffered formalin for 48 hr and embedded in paraffin. Multiple sections (4 μm thick) were deparaffinized with xylene and stained with hematoxylin and eosin (H&E) or Sirius red stain to detect collagen. Immunohistochemistry was carried out in the same sections using primary mouse antibody to 8-hydroxy-deoxyguanosine (8-OHdG; 1:200, QED Bioscience, San Diego, CA, USA, 12501) and primary rabbit antibody Ki-67 (1:100, Abcam, Cambridge, UK, Ab16667). The Dako animal research kit based on avidin–biotin and peroxidase methodology was used for mouse primary antibodies (Dako Diagnóstico S.A., Madrid, Spain) and Vectastain ABC kit for rabbit primary antibodies (Vector PK6101; Burlington, NC, USA). The peroxidase substrate DAB kit (Vector SK-4100) was used for qualitative identification of antigens by light microscopy. Apoptotic cells were detected using a TUNEL kit (ApopTag plus Peroxidase in Situ Apoptosis Detection Kit, Millipore, Billerica, MA, USA, Code: S7101). Sections were examined with an OLYMPUS CX41 microscope, and images were scanned under equal light conditions using the CELL A program (Olympus, Hicksville, NY, USA).

8-OHdG in epithelial cells was quantified in all layers, and for Ki-67, the intensity per nucleus was counted. The epithelia region was selected on the basis of its histological appearance, and the same region was used for all immunohistochemistry studies. Values included at least 500 cells from four different rats (at least five different images each). In every image, the number of Ki-67- and 8-OHdG-positive cells was calculated as the number of + cells/mm^2. A similar method was used to count the number of TUNEL-positive cells, which were quantified as + cells/mm^2, and included all epithelial layers and stroma above the muscle layer. For collagen, we measured the extent of Sirius red staining between basal epithelial cells and lingual muscle. Values correspond to the average of different images from four different rats (at least five images from each rat).

Isolation of pure mitochondria, nuclei, and cytosol from the tongue

Animals were sacrificed, and tongue mitochondria, nuclei, and cytosol immediately isolated by differential centrifugation and Percoll density gradient as described previously [19].

Measurement of LPO and GPx and GRd activities

Lipid peroxidation (LPO) was assessed using a commercial kit (Bioxytech LPO-568 assay kit; OxisResearch, Portland, OR, USA) [20]. Glutathione peroxidase (GPx) and glutathione reductase (GRd) activities were spectrophotometrically measured [21] in a UV spectrophotometer (model UV-1603, Shimadzu Deutschland GmBH, Duisburg, Germany).

Measurement of GSH and GSSG levels

Glutathione (GSH) and glutathione disulfide (GSSG) were measured using an established fluorometric method [22] in a plate-reader spectrofluorometer (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Determination of mitochondrial complex activity

The activity of mitochondrial complexes was spectrophotometrically measured (model UV-1603; Shimadzu Deutschland GmBH) in pure mitochondrial fractions. Complex I (NADH CoQ oxidoreductase, expressed as nmol oxidized NADH/min/mg prot) activity was measured by following the oxidation rate of NADH at 340 nm [23]; complex II (succinate: DCIP oxidoreductase, expressed as nmol reduced DCIP/min/mg prot) activity by following the rate of 2.6-DCIP reduction at 600 nm with 520 nm [24]; complex III (ubiquinol: cytochrome c reductase, expressed as nmol reduced cytochrome c/min/mg prot) activity by following the rate of cytochrome c reduction at 550 nm [24]; and complex IV (cytochrome c oxidase, expressed as nmol oxidized cytochrome c/min/mg prot) activity by following the reduction of cytochrome c previously reduced with sodium borohydride [23].

Citrate synthase activity

Citrate synthase activity was determined in mitochondria spectrophotometrically (model UV-1603; Shimadzu Deutschland GmBH) at 30°C [25].

Mitochondrial respiration

Function of isolated mitochondria was assessed using high-resolution respirometry (Oroboros Instruments, Innsbruck, Austria) in an oxygraph equipped with a polarographic oxygen sensor [26]. Isolated mitochondria were gently re-suspended in MiR05 medium (0.5 mM EGTA, 3 mM $MgCl_2.6H_20$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-sucrose, 1 g/L bovine serum albumin, essentially fatty acid free, 60 mM lactobionic acid, pH 7.1) at 37°C. A total of 0.017 mg/mL of isolated mitochondria was added to 2 mL MiR05 medium in each chamber of the oxygraph. Substrates for complex I and complex II were used in two different protocols to determinate mitochondrial function. Protocol 1 evaluated representative traces for the complex I assessment with

glutamate (10 mM) and malate (2 mM) as substrates. ADP-stimulated respiration (state 3) was measured with 0.25 mM ADP. Leak state was obtained after inhibition of ATP synthase with oligomycin (3 μg/mL). Electron transfer system (ETS) capacity was induced experimentally by 0.5 μM titrations of carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP). Residual oxygen consumption was used to correct all values by adding inhibitors of complex I (2.5 μM rotenone), complex II (5 mM malonate), and complex III (2.6 μM antimycin A). Protocol 2 was used to evaluate the mitochondrial function of complex II linked with succinate (as substrate for complex II) plus rotenone. ADP, oligomycin, FCCP titration, malonic acid, and antimycin A were added in the same concentrations as in protocol 1. All values were expressed in pmol/(s $\times$ mg protein).

Reverse transcription–polymerase chain reaction

RNA was isolated using the Real Total Spin Plus kit (Durviz, S.L., Valencia, Spain). RT-PCR was performed in a Stratagene 3005P system as described previously [27] and normalized to GADPH. Primer sequences (Table S1) were designed using Beacon Designer software (Premier Biosoft Int., Palo Alto, CA, USA).

Western blot analysis

We performed protein extraction and Western blot analyses as described previously [27]. The antibodies used in this study included rabbit antibody to GPx (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA, SC30147), GRd (1:200, Santa Cruz Biotechnology, SC32886), Mn-SOD (1:500; Santa Cruz Biotechnology, SC30080), p53 (1:200; Santa Cruz Biotechnology, SC6243), Bax (1:200; Santa Cruz Biotechnology, SC526), Bcl2 (1:200; Santa Cruz Biotechnology, SC492), ASC (1:1000; AdipoGen, San Diego, CA, USA, AG-25B-0006-C100), caspase 1 (1:500; Santa Cruz Biotechnology, SC22165), IL-1β (1:100; Santa Cruz Biotechnology, SC7884), Beclin-1 (1:500; Cell Signaling Technology, Beverly, MA, USA, 3738), Nix (1:500; Sigma, Madrid, Spain, N0399), NRF1 (1:200; Santa Cruz Biotechnology, SC33771), PGC1-α (1:200; Santa Cruz Biotechnology, SC13067), TFAM (1:100; Abnova, Walnut, CA, USA, H00007019-d01P), iNOS (1:100; Santa Cruz Biotechnology, SC650), nitrotyrosine (1:250; Calbiochem, San Diego, CA, USA, 487924), COX-2 (1:200; Santa Cruz Biotechnology, SC7951), and ROR-γ (1:200; Santa Cruz Biotechnology, SC28559); mouse antibody to OXPHOS (MitoProfile Total OXPHOS Rodent WB Antibody Cocktail; 1:250; Mitosciences, Eugene, OR, USA, MS604), cytochrome c (1:500; Mitosciences, MS604), caspase 3 (1:200; Santa Cruz Biotechnology, SC136219), NLRP3 (1:500; AdipoGen, AG-20B-0014), Atgl2 (1:100; Santa Cruz Biotechnology, SC271688), sirtuin (1:500; Millipore, 04-1557), NFκB (p65) (1:100; Santa Cruz Biotechnology, SC8008), IκBα (1:200; Santa Cruz Biotechnology, SC1643), and TNFα (1:200; Santa Cruz Biotechnology, SC52746); goat antibody to pro-caspase 1 (1:500; Santa Cruz Biotechnology, SC622), MT1 (1:200; Santa Cruz Biotechnology, SC13186), and MT2 (1:200; Santa Cruz Biotechnology, SC13177); goat anti-rabbit IgG (1:5000; Thermo Scientific Pierce, Rockford, IL, USA, Antibody 31460); HRP goat anti-mouse IgG (1:1000; BD Pharmingen, San Jose, CA, USA 554002); and donkey anti-goat IgG-HRP (1:1000; Santa Cruz Biotechnology, SC2020). The proteins were visualized using a chemiluminescence kit (Western Lightning Plus-ECL; PerkinElmer, Billerica, MA, USA) according to the manufacturer's protocol. Images were analyzed using a Kodak image analysis station (Image Station 2000R; Eastman Kodak Company, Rochester, NY, USA).

Quantification of CoQ9 and CoQ10 levels in tongue

Coenzyme Q9 (CoQ9), the major form of ubiquinone in rodents, and coenzyme Q10 (CoQ10) were extracted with 1-propanol. Samples were analyzed using HPLC (Gilson, Middleton, WI, USA), and the lipid components separated using a reverse-phase symmetry C18 column (3.5 μm, 4.6 $\times$ 150 mm; Waters, Barcelona, Spain). The electrochemical detector consisted of an ESA Coulochem III with a guard cell (upstream of the injector) at +900 mV and conditioning cell at $-$600 mV (downstream of the column) followed by the analytical cell at +350 mV [28].

Determination of melatonin by HPLC

Melatonin was extracted with trichloromethane and the organic phase evaporated to dryness (SPD 2010 Speed Vac System; Fisher Scientific, Madrid, Spain). Samples were analyzed using HPLC (Shimadzu Europe GmbH, Duisburg, Germany) with a Waters Sunfire C18 column (150 $\times$ 4.5 mm, 5 μm). Melatonin fluorescence was measured in a fluorescence detector (Shimadzu RF-10A XL fluorescence detector; Shimadzu Europe GmbH, Duisburg, Germany) with excitation and emission wavelengths of 285 and 345 nm, respectively [29].

Liquid chromatography–mass spectrometer (UPLC/MS/MS) analysis of melatonin metabolites

N-acetyl-5-methoxykynuramine (AMK) and N-acetyl-N-formyl-5-methoxykynuramine (AFMK) were extracted using the same method as for melatonin [30]. AMK and AFMK were analyzed using a Waters Acquity H-Class UPLC (Milford, MA, USA) coupled to a Xevo TQ S mass spectrometer (MS/MS) with electrospray ionization (Waters Corporation). A Waters Acquity ethylene-bridged (BEH) C18 column (100 $\times$ 2.1 mm, 1.7 μm) was used with an oven temperature of 40°C. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B was acetonitrile with 0.1% formic acid. Separation was achieved using a gradient program [30] at a flow rate of 0.4 mL/min. The capillary voltage and extractor voltage were 2.5 kV and 4 V, respectively; the flow rate of the cone gas and desolvation gas was 150 and 800 L/hr, respectively, and the source temperature and desolvation temperatures were 150 and 350°C, respectively. Data were acquired with MassLynx 4.0 software and calibrated and quantified by QuanLynx software.

Statistical analysis

Groups were compared using two-tailed unpaired Student's *t*-tests in Prism software (GraphPad, La Jolla, CA, USA). Data are presented as mean ± S.E.M. Differences were considered statistically significant at $P < 0.05$.

Results

To determine whether melatonin counteracts the deleterious effects of radiation in normal epithelial cells of the tongue and the dose required to achieve this result, we applied 1%, 3%, or 5% melatonin gel solution (w/v) to the oral mucosa of irradiated rats. A maximal effect was obtained with 3% melatonin gel, corresponding to 45 mg oral melatonin/day/rat, based on the prevention of cellular and mitochondrial LPO (Fig. 1B). Thus, 3% melatonin gel was used in all subsequent experiments and its effects compared to animals treated with 45 mg melatonin/day/rat administered ip. Macroscopic (Fig. 1C) and histological (Fig. 2A,D) analyses of tongues revealed typical signs of mucositis in irradiated animals, which were absent in melatonin gel-treated rats. The IR group exhibited a loss of filiform papillae, ulcerations in the mucosal lining, disruption of the epithelium layer, inflammatory cell infiltration, congestion of blood vessels in the submucosa, increased submucosal thickness, and decreased mucosal thickness. The ip aMT group exhibited augmented submucosal thickness with a slight increase in blood vessels. Extensive ulceration was absent in the melatonin gel group. Treatment with melatonin gel prevented excess collagen production (Fig. 2B,E) in the oral mucosa of irradiated rats. In contrast, ip aMT treatment caused minor improvements in the signs of mucosal degeneration (Fig. 2B,E). Importantly, the protection conferred by melatonin treatment may be complete only at doses achieved with topical gel application.

Both epidermis and mucosal epithelia depend on resident self-renewing stem cells, making them particularly vulnerable to radiotherapy [31]. Immunostaining with specific antibodies against Ki-67, a marker of proliferating cells, revealed a significant reduction in proliferating cells in untreated tongues with mucositis (Fig. 2C,F). We also found higher cell proliferation in tongues treated with melatonin gel than in tongues treated with ip aMT (Fig. 2C,F). These results reflect the ability of melatonin to prevent the loss of proliferative progenitor stem cells upon radiation, enhancing their capacity to repopulate the tissue.

Mitochondrial damage could be involved in radiation-induced mucosal injury [32]. We found that radiation increased the activity and protein levels of mitochondrial GPx (Fig. 3A) and GPx mRNA levels (Figure S1A), whereas the activity and expression of GRd were significantly reduced compared to nonirradiated controls (Fig. 3B). These findings reflect an oxidizing response of mitochondria to radiation, which was further supported by increased mitochondrial GSSG/GSH ratio (Fig. 3C). However, radiation did not affect the activity or expression of mitochondrial superoxide dismutase (Mn-SOD) (Figure S1C). Application of melatonin gel prevented mitochondrial hyperoxidative status in the tongues of irradiated rats, recovering the normal GSSG/GSH ratio; this effect was less pronounced with ip aMT treatment (Fig. 3A–C and Figure S1A,B).

Mitochondrial oxidative stress may affect bioenergetic capacity. We found a significant reduction in the expression of respiratory complexes I, III, and IV and the ATPase (Fig. 3D), which was reflected primarily in reduced complex I and IV activities (Figure S1D). We also detected a reduction in mitochondrial mass as a reduction in citrate synthase activity (Fig. 3E) and a decrease in the ETS capacity through complex I in the mitochondria of irradiated rats (Fig. 3F), without effects through complex II (data not shown). Melatonin gel application completely counteracted mitochondrial dysfunction (Fig. 3A–F). Moreover, although irradiation did not cause significant changes in CoQ9 content, melatonin gel treatment significantly increased CoQ9 in irradiated rats (Fig. 3G). Together, these results support the efficacy of melatonin gel to boost mitochondrial function in irradiated rats, which could prevent energy depletion and subsequent cell death. These data related to the bioenergetics of mitochondria in mucositis have not been reported previously, and they support the typical effects of melatonin in the maintenance of mitochondrial homeostasis [13, 33].

Although the mechanisms involved in radiotherapy-mediated cell apoptosis are still poorly understood, they probably involve ROS production [34] and subsequent DNA degradation. Therefore, we investigated whether these mechanisms underlie the protective effect of melatonin against radiation. As expected, radiation increased 8-OHdG levels in the tongue (Fig. 4A,B), which correlated with a marked increase in the number of apoptotic cells (Figure S2A,B). Next, we evaluated the mechanism(s) of apoptosis and found that irradiation upregulated p53 and Bax and downregulated Bcl2 mRNA and protein levels, increasing the Bax/Bcl2 ratio (Fig. 4C and Figure S2C). These conditions favor the opening of the mitochondrial permeability transition pore, releasing cytochrome c to the cytosol (Fig. 4D) and activating caspase-3 (Fig. 4E). To compensate for the loss of cytochrome c inside the mitochondrion, radiation induced its expression (Figure S2D). Of note, melatonin gel administration inhibited all of the events in mitochondrial-dependent apoptosis described here, but ip aMT did so only partially.

Defects in mitochondrial function and subsequent bioenergetic failure are not only linked to apoptosis but also related to the induction of mitochondrial autophagy (mitophagy) to remove dysfunctional mitochondria [35]. Although autophagy is not involved in cell death, in some conditions, it is concurrent with or sequential to apoptosis, cooperating in cell death [36]. Western blot analysis revealed an increase in autophagy/mitophagy markers Atg12, Beclin-1, and Nix in irradiated tongues, which was inhibited by melatonin (Figure S3A). This result aligns with the observation that melatonin reduced mitochondrial dysfunction in oral mucosa and could thus reduce mitochondrial damage. To confirm this hypothesis, we used EM to analyze mitochondrial structure (Fig. 5). In the tongues of irradiated rats, we observed cells with numerous broken and swollen mitochondria, poor cristae,

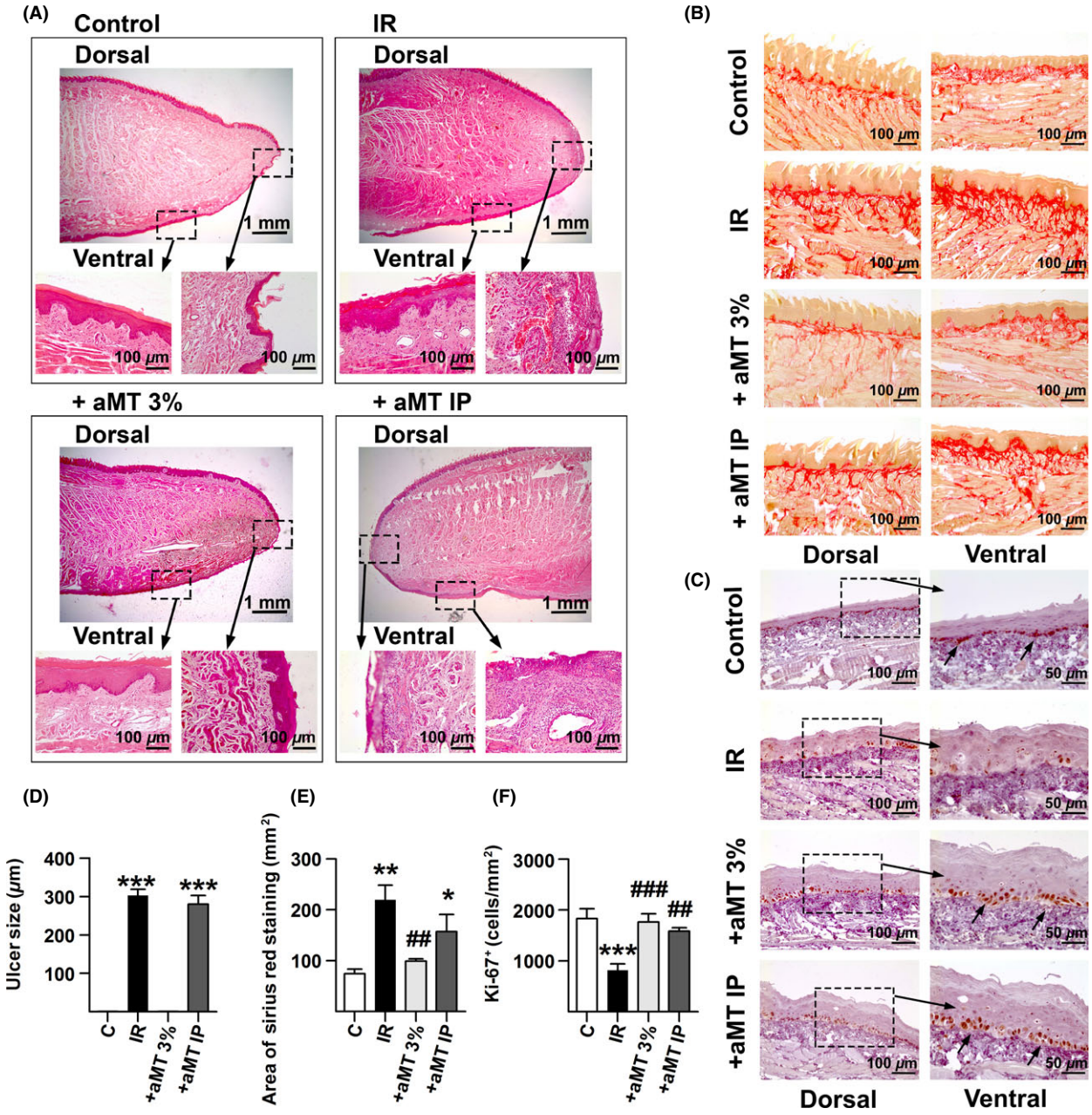

Fig. 2. Melatonin gel 3% prevents radiation-induced oral mucositis in rats. (A) H&E staining of the tongue from control (nonirradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). The different groups show dorsal and ventral epithelial morphology. The small panels show details at higher magnification. Scale bar = 1 mm and 100 μm in the upper and lower panels, respectively. (B) Sirius red staining to detect collagen production in ventral (right panel) and dorsal (left panel) tongue sections. Scale bar = 1 mm and 100 μm in the upper and lower panels, respectively. (C) Immunohistochemical staining of the proliferation marker Ki-67 in ventral tongue section. The right panels show details at higher magnification. Scale bar = 100 μm and 50 μm in the left and right panels, respectively. (D) Tongue ulcer sizes corresponding to H&E staining. (E–F); quantification of the staining in B and C, $n = 4$ per group. Data are mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

and numerous vacuoles. In addition, muscle fibers were disorganized. The administration of melatonin gel completely prevented this radiation-induced mitochondrial disintegration, but ip aMT had only a minor effect (Fig. 5). Defects in mitochondrial function could be associated with increased mitochondrial biogenesis to replace damaged mitochondria. We found increased protein and mRNA levels of transcription factors NRF-1, PGC1α, TFAM, and SIRT 1 in all irradiated groups (Figure S3B). However, changes in these transcription factors did not precede any increase in mitochondrial mass as assessed by the reduction in citrate synthase activity in these irradiated rats (Fig. 3E), possibly because of continuous oxidative damage and mitophagy during irradiation. Preventing mitochondrial ROS and preserving NRF-1, PGC1α, TFAM, and SIRT 1 expression with melatonin treatment

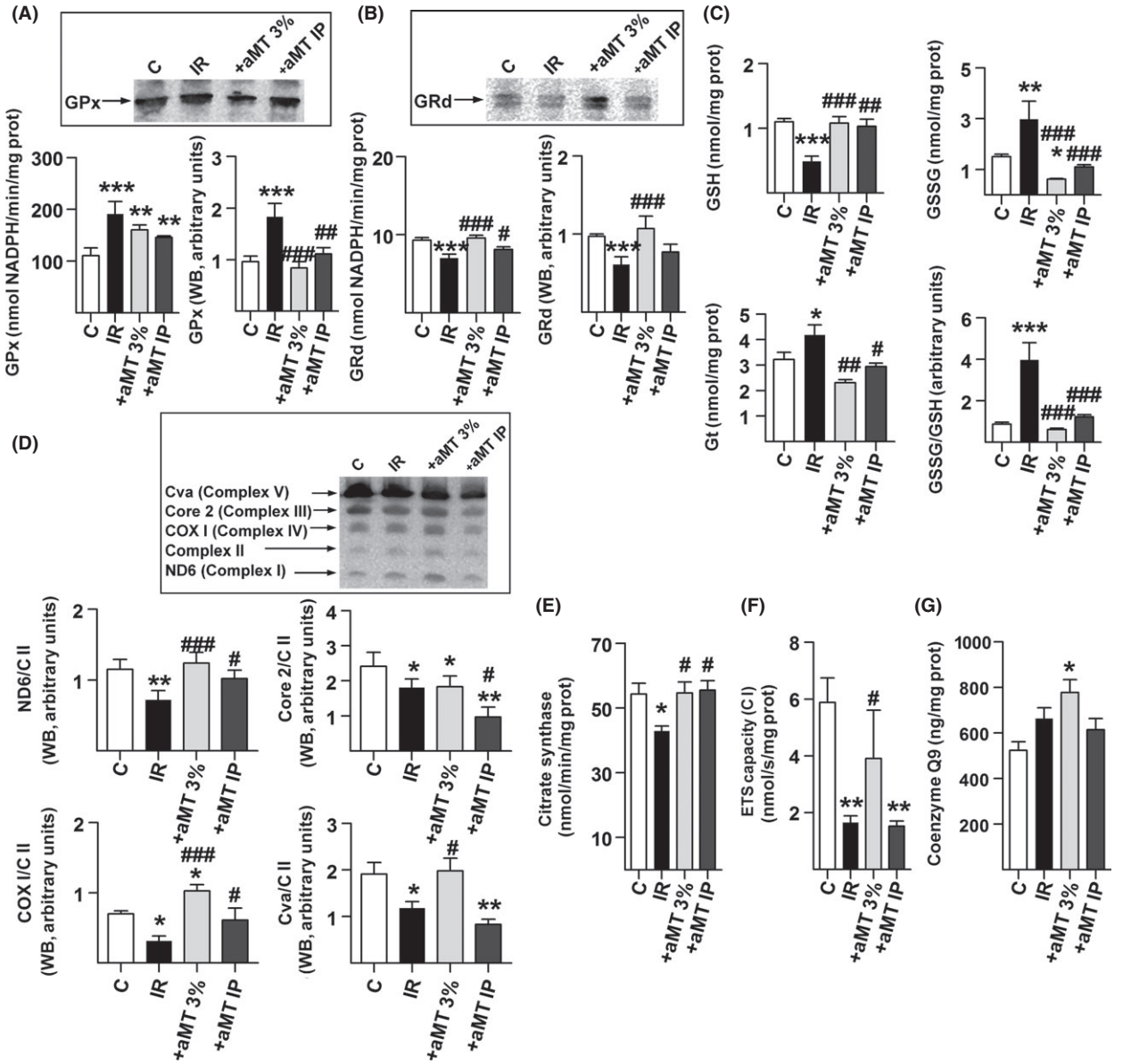

Fig. 3. Melatonin gel 3% prevented mitochondrial dysfunction in irradiated rats. (A) and (B), Activity and Western blot (WB) analysis of GPx (A) and GRd (B) in the mitochondria of tongue cells from control (nonirradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). Western blots were quantified by densitometry. (C) GSH levels and GSSG/GSH ratio in the mitochondria of tongue cells. (D) WB analysis and densitometric quantification of OXPHOS in the mitochondria of tongue cells, $n = 6$ per group. (E) Citrate synthase activity in tongues. (F) Mitochondrial respiration as represented by the ETS capacity through complex I in the mitochondria of tongue cells. $n = 6$ per group. (G) HPLC analyses of coenzyme Q9 levels in tongues, $n = 4$ per group. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

during irradiation could allow the maintenance of physiological mitochondrial mass (Fig. 3E).

We next asked whether mitochondrial dysfunction during irradiation can activate the NLRP3 inflammasome, which would explain the lack of response of mucositis to anti-inflammatory drugs [1]. Our results revealed a significant increase in the NLRP3 and ASC (apoptosis-associated speck-like protein containing CARD, caspase activation, and recruitment domain) protein levels and their corresponding mRNA expression in the tongues of irradiated rats compared to nonirradiated controls (Fig. 6A). The expression of pro-caspase-1 mRNA increased with irradiation, but its protein levels decreased, reflecting its activation to caspase-1 by activated NLRP3 (Fig. 6A,B), which yielded a significant increase in IL-1β (Fig. 6C). Thus, dysfunctional mitochondria trigger the activation of the NLRP3 inflammasome, which depends on mitochondrial ROS production, among other events [37]. These results support NLRP3 as a new player in the pathogenesis of irradiation-induced mucositis. Therefore, scavenging ROS and protecting mitochondria from irradiation constitute two important mechanisms to suppress

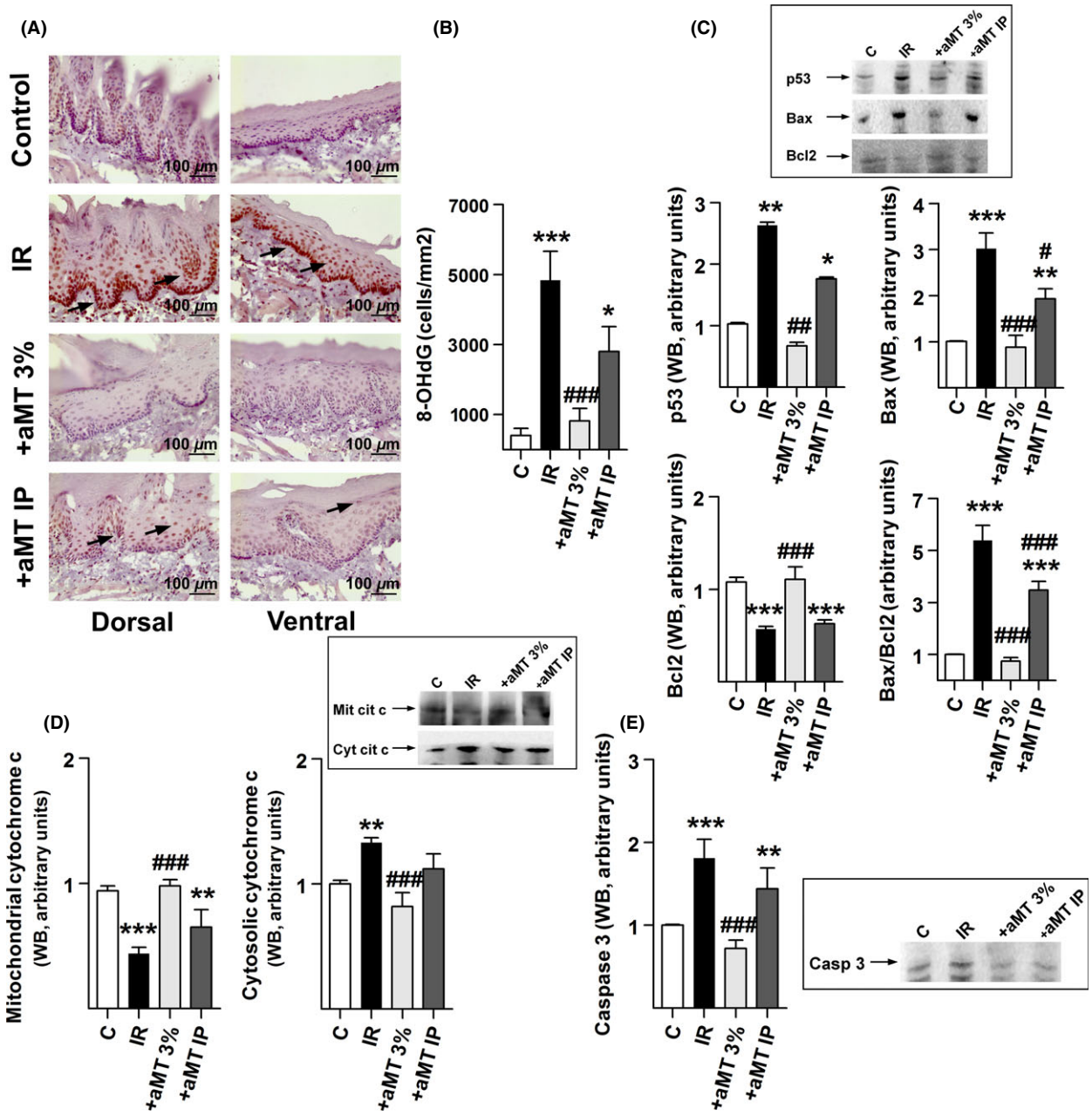

Fig. 4. Melatonin gel 3% prevented apoptosis in irradiated rats. (A) Immunohistochemical staining of 8-OHdG in sections of dorsal and ventral tongue from control (nonirradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). Scale bar = 100 μm. (B) Quantification of the staining in A, $n = 4$ per group. (C–E) WB analysis and densitometric quantification of p53, Bax, and Bcl2, and Bax/Bcl2 ratio in tongues (E); cytochrome c in the mitochondrial and cytosolic fractions of tongue cells (D); and caspase 3 in tongues (E), $n = 6$ per group. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

inflammasome activation in melatonin-treated irradiated rats.

Because NLRP3-activated caspase-1 requires pro-IL-1ß as a substrate, which is produced by the NF-κB pathway, we evaluated the contribution of the NF-κB pathway to irradiation-induced oral mucositis [34, 38]. We observed a significant elevation in NF-κB mRNA and protein levels in the cytosol and nuclei of irradiated rat tongue cells (Fig. 7A and Figure S4A). Parallel changes were observed in the expression of the inhibitory protein IκB, which is under NF-κB control (Fig. 7B and Figure S4B). Only local application of melatonin gel suppressed the irradiation-activated NF-κB pathway (Fig. 7A and Figure S4A) and increased IκB mRNA and protein levels (Fig. 7B and Figure S4B). These results are consistent with the ability of oral melatonin to inhibit NF-κB signaling [39].

To determine the functional importance of the NF-κB-dependent innate immune response in our experi-

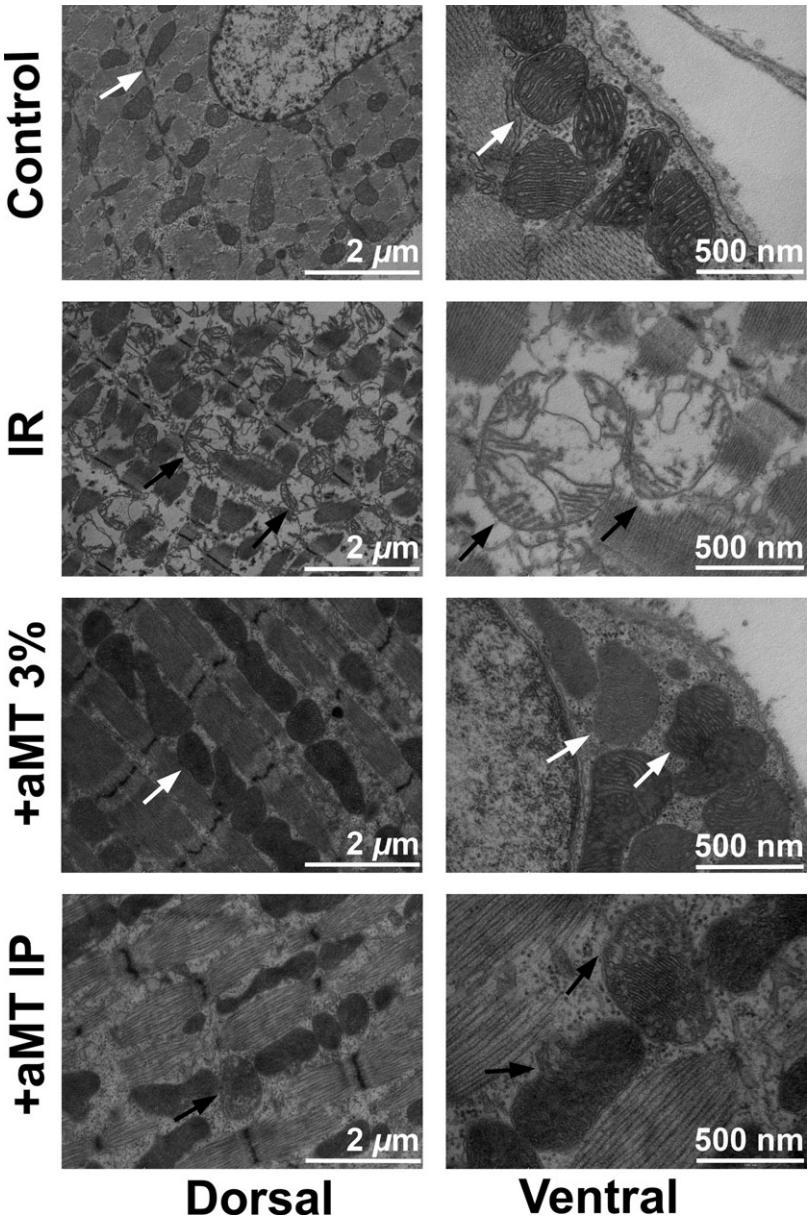

Fig. 5. Electron microscopy (EM) analysis of tongue tissue. EM images of mitochondria in tongues from control (nonirradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). $n = 4$ per group. Swollen and less dense mitochondria are indicated by black arrows; normal mitochondria are indicated by white arrows. Right panels show details at higher magnification. Scale bar = 2 μm and 500 nm in the left and right panels, respectively.

mental model, we analyzed the transcription of NF-κB-dependent genes involved in mucositis [34]. Our study confirmed increased protein (Fig. 7C) and mRNA levels (Figure S4C) of pro-IL-1β and TNFα in the tongues of irradiated rats. TNFα and activated IL-1β collaborate to induce inducible nitric oxide synthase (iNOS), yielding high amounts of nitric oxide (NO) [40]. Although the role of NO in mucositis is not fully understood, iNOS and mitochondrial iNOS (i-mtNOS) expression increased by the inflammatory response [9] (Fig. 7D and Figure S4D) and increased levels of nitrosylated proteins (Fig. 7E) in the tongues of irradiated rats support the contribution of NO to mucosal damage. Irradiation also significantly induced COX-2, another mediator of the NF-κB response (Fig. 7F and Figure S4E).

Local application of melatonin gel in the rat's mouth inhibited the irradiation-induced NF-κB-dependent innate immune response, including the expression of inflammatory mediator mRNA and protein. However, the anti-inflammatory efficacy of melatonin was much less significant with systemic administration (Fig. 7 and Figure S4). Overall, the results consistently support that the melatonin gel ameliorates inflammation, blocking NF-κB [41, 42] as well as the inflammatory mediators COX-2 and iNOS, which are molecular targets for melatonin and its metabolites [9, 39].

To determine whether melatonin reaches the oral mucosa and protects it against radiation in situ, we assessed the indoleamine content of rat tongue. Radiation drastically reduced melatonin levels in untreated tongue (Fig. 8A), suggesting that it induced a rapid consumption of melatonin, probably because of free radical scavenger activity [43]. Supporting this hypothesis, we found that AFMK and AMK, the two main melatonin metabolites, increased significantly in irradiated tongues (Fig. 8B). AFMK is produced when melatonin interacts with ROS and is converted to AMK, which can also scavenge ROS [44]. Nevertheless, the AFMK/aMT ratio was almost 2 in

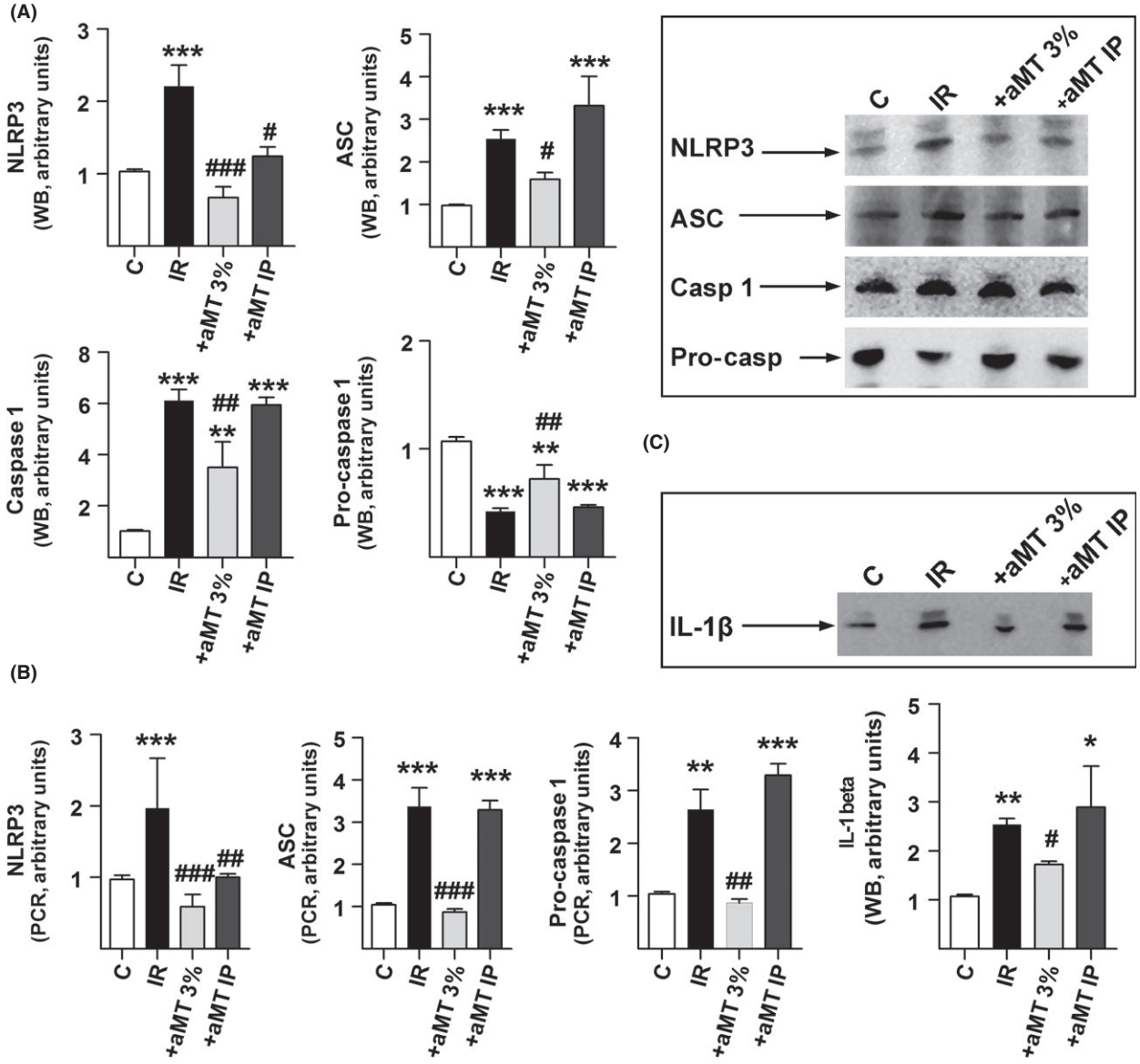

Fig. 6. NLRP3 inflammasome pathway inhibition by melatonin gel 3% during irradiation. (A) Western blot (WB) analysis and densitometric quantification of NLRP3, ASC, pro-caspase 1, and caspase 1 in tongues from control (nonirradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). (B) Quantitative RT-PCR of NLRP3, ASC, and pro-caspase 1 in tongues. (C) WB analysis and densitometric quantification of caspase 3 in tongues, $n = 6$ per group. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

normal tongue and increased up to 50 after irradiation, suggesting an additional source of melatonin. Two main enzymes control melatonin synthesis: arylalkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT). ASMT is likely the limiting enzyme in this pathway instead of AANAT [19]. Accordingly, we found enhanced expression of ASMT mRNA in irradiated tongues (Fig. 8C), which explains the increased melatonin production with radiation, followed by rapid metabolization to AFMK and AMK. Our results give the first indication of melatonin production in oral mucosa and demonstrate that only treatment with aMT gel restores basal AANAT and ASMT mRNA expression in irradiated rats, recovering the normal equilibrium between melatonin and its metabolites (Fig. 8A–C).

We also explored the participation of melatonin receptors in the prevention of oral mucositis by melatonin gel. Our results confirm the presence of membrane receptors MT1 and MT2 in rat tongue; their expression, particularly that of MT1 mRNA and protein, was reduced with radiation (Fig. 8D–E). We also provide the first evidence of RORγ in rat tongue (Fig. 8F), but not RORα or RORβ, nuclear receptor subtypes involved in some of the genomic actions of melatonin; irradiation also reduced RORγ (Fig. 8F). Treatment with melatonin gel recovered the expression and protein levels of MT1, MT2, and RORγ receptors (Fig. 8D–F). Interestingly, although these receptors had a K_d of melatonin in the picomolar to nanomolar range, our results suggest that high doses of melatonin, such as those used here for therapeutic purposes, may

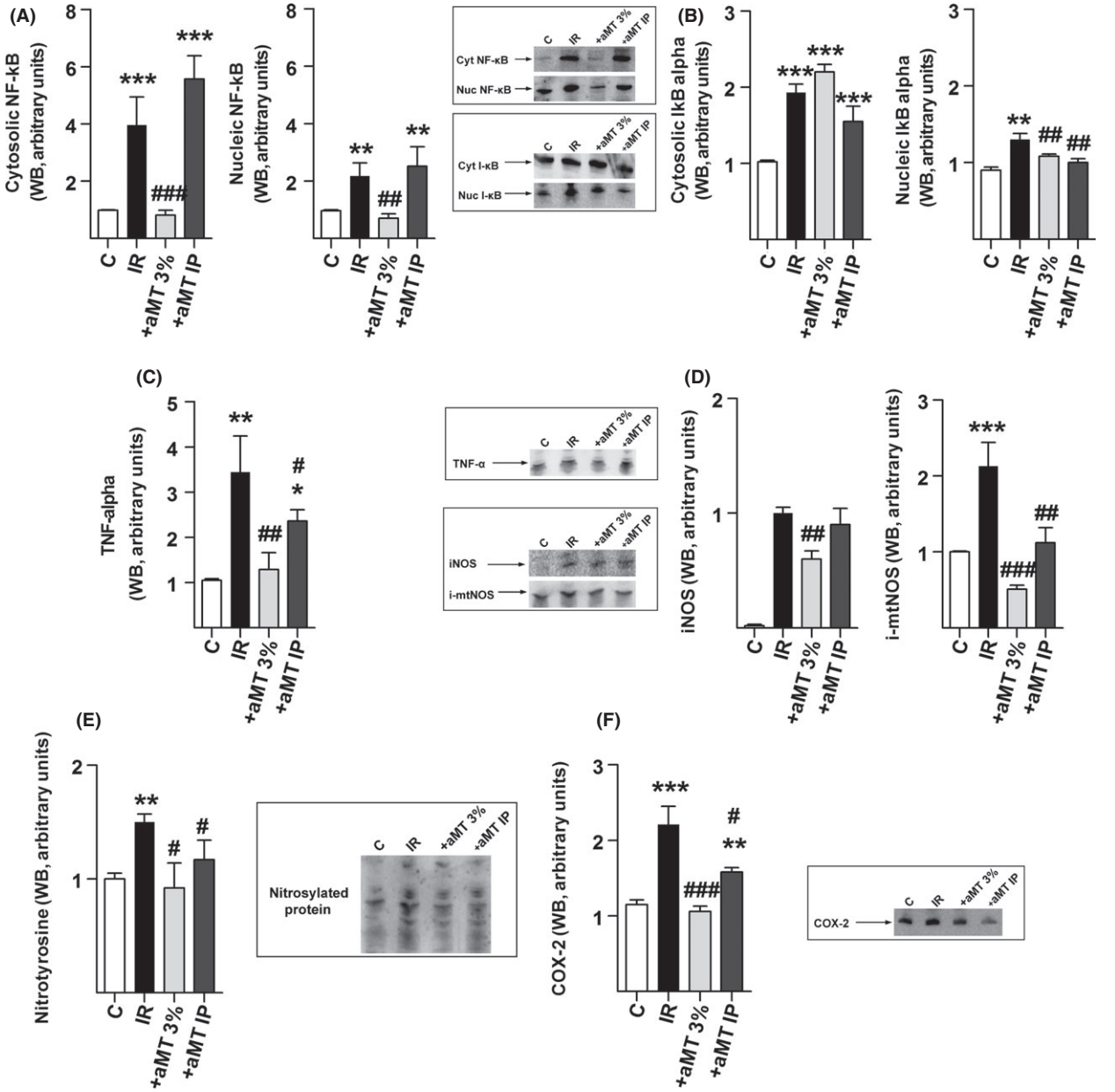

Fig. 7. Melatonin gel 3% inhibits radiation-induced NF-κB pathway activation. (A–F) Western blot (WB) analysis and densitometric quantification of NF-κB subunit p65 in nuclear and cytosolic fractions (A); IκB-α, inhibitor of p65 active subunit of NF-κB, in nuclear and cytosolic fractions (B); TNF-α in cells (C); cytosolic iNOS and mitochondrial iNOS (i-mtNOS) in cells (D); nitrotyrosine residues in cellular proteins (E); cytosolic COX-2 in cells (F) of tongue from different groups: control (nonirradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP), $n = 6$ per group. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

exert additional effects through melatonin receptors because of their increased expression [27].

As a control for the effects of melatonin, a group of rats was treated with melatonin gel in the absence of irradiation. The rats underwent the same treatment scheme as shown in Fig. 1A, but they were not irradiated. No differences were observed in response to melatonin gel in nonirradiated rats compared to untreated nonirradiated rats (Figure S5). However, the melatonin content was higher in the tongues of nonirradiated rats treated with melatonin gel compared to irradiated and treated rats, suggesting a lack of melatonin consumption in the absence of oxidative damage (Figure S5I).

Discussion

Emerging evidence supports the activation of the NLRP3 inflammasome being triggered by dysfunctional mitochondria, dependent on mitochondrial ROS production [7, 37]. Here, we show that in addition to the NFκB pathway, mitochondrial dysfunction and subsequent NLRP3 inflammasome activation are two main players involved

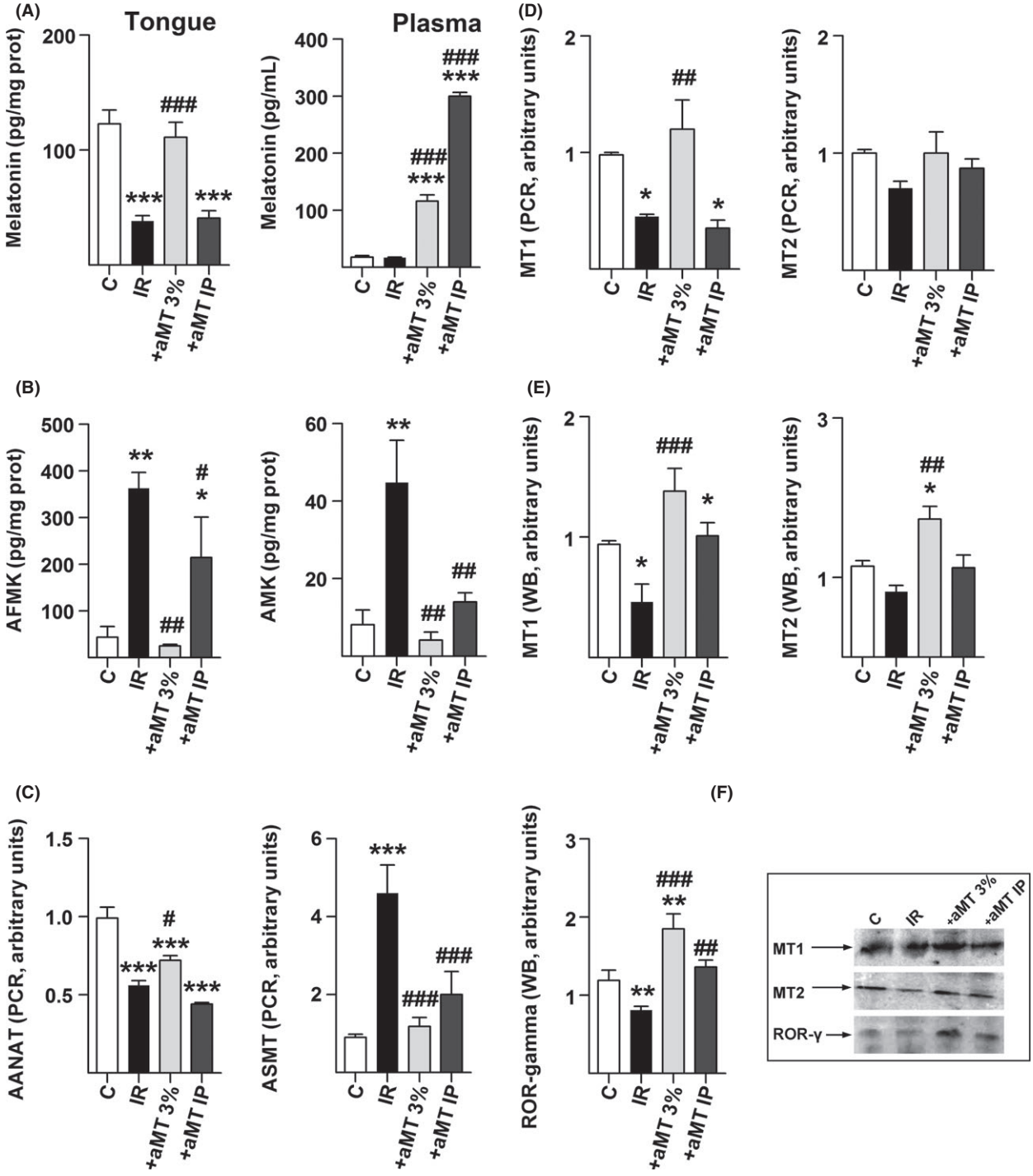

Fig. 8. Alterations in the melatonin pharmacodynamics of irradiated tongues. (A) HPLC analysis of the melatonin content of tongues and plasma from control (nonirradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP), $n = 6$ per group. (B) Mass spectrometric analysis of the melatonin metabolites AFMK and AMK in tongues, $n = 4$ per group. (C) Quantitative RT-PCR of AANAT and ASMT in tongue. (D) Quantitative RT-PCR of MT1 and MT2 melatonin membrane receptors in tongue. (E) and (F), Western blot (WB) and densitometric quantification of MT1 and MT2 receptors (E) and RORγ nuclear receptor of aMT (F) in tongues, $n = 6$ per group. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

in the development of oral mucositis during irradiation. Therefore, a connection between mitochondrial impairment and innate immune activation underlies irradiation-induced oral mucositis. We also report an effective treatment against irradiation-induced mucositis, a topical application of a melatonin gel that reduces the development and extent of erythema and prevents ulcer formation.

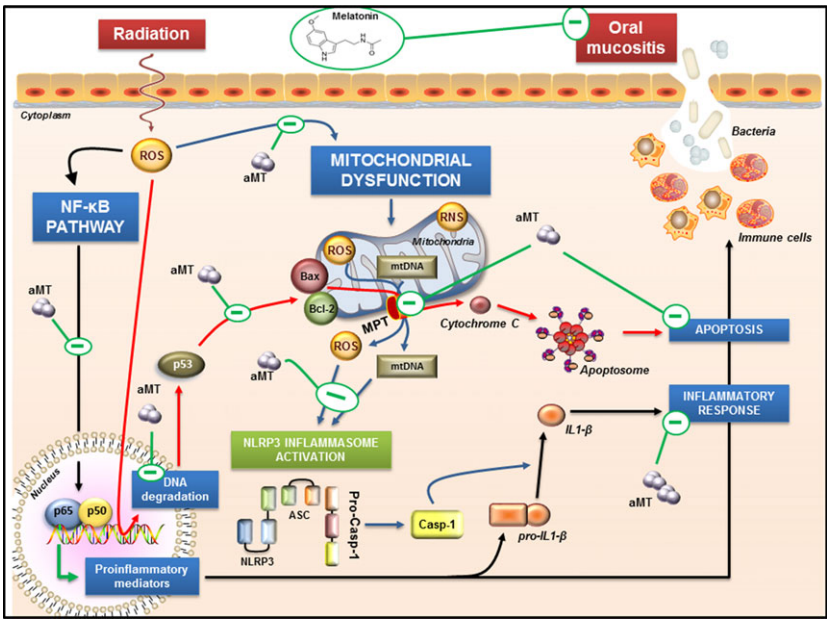

Fig. 9. The molecular pathway involved in radiotherapy-induced mucositis that is inhibited by melatonin gel. Radiation-induced ROS activate several pathways that converge in oral mucositis. ROS activate the NF-κB pathway, including pro-inflammatory cytokines and enzymes. In addition, iNOS, pro-IL-1β, ROS, and the inflammatory response induce mitochondrial dysfunction and apoptosis. Moreover, impaired mitochondria release ROS, which activates the NLRP3 inflammasome pathway. NLRP3 activates caspase-1, which produces mature IL-1β. Together, NF-κB and NLRP3-dependent inflammation and apoptosis cause oral mucositis. Melatonin gel protects mitochondria and inhibits the NF-κB and NLRP3 inflammasome pathways, reducing inflammation and apoptosis and preventing the development of oral mucositis.

The mitochondria in rat tongue enter a hyperoxidative status following exposure to ionizing radiation, resulting in impaired mitochondrial bioenergetics. To remove damaged mitochondria and likely to prevent apoptosis [36], the cell triggers autophagy/mitophagy. This response, however, is not effective, and autophagy and apoptosis occur together in the irradiated oral mucosa. Autophagy can facilitate apoptosis by maintaining ATP levels during starvation to promote ATP-dependent apoptotic processes [36]. Moreover, impaired irradiated mitochondria also release ROS to the cytosol, activating the NLRP3 inflammasome [7, 37, 45]. This alternative but complementary pathway of the innate immune system activates pro-inflammatory cytokines produced by activation of the NF-κB pathway [8]. The connection between the—NF-κB and NLRP3—pathways creates a vicious cycle during irradiation, resulting in the transcriptional upregulation of many genes involved in mucositis [34]. Our results indicate that NF-κB and NLRP3 work together to activate the inflammatory pathways of the innate immune response, resulting in an overstimulation [46]. Excessive inflammation in turn creates a microenvironment suitable for oral mucositis [47]. The participation of the double innate immune responses explains the lack of effective therapy against mucositis, but the impaired mitochondria underlying these inflammatory responses make a specific treatment more difficult.

We selected melatonin as a potential therapy because of its role as a cell protector in the body [13, 33]. Moreover, most organs and tissues of the body produce melatonin [48, 49]. The efficacy of melatonin depends on its ability to completely prevent the activation of multiple irradiation-induced pathways identified here as being associated with mucositis. The antagonist effect of melatonin on both NF-κB and NLRP3 signaling could make it a more efficient anti-inflammatory molecule than agents targeting only NF-κB. The therapeutic benefit of melatonin gel against oral mucositis may also be attributed to its enhanced capabilities to protect mitochondria in epithelial cells, as well as stem cells, from radiation.

We also showed for the first time that melatonin is synthesized in the rat tongue, where it acts in autocrine/paracrine signaling by binding to MT1, MT2, and RORγ, suggesting a role in normal oral mucosal physiology. Although it is well known that skeletal muscle and skin contain melatonin [50, 51], this is the first time reporting the production of melatonin by the oral mucosa. However, irradiation drastically decreased basal melatonin levels, impeding its protective role. Interestingly, the induction of MT1/MT2 melatonin membrane receptor expression after high-dose melatonin administration indicates that the therapeutic benefits of melatonin are not restricted to low doses. These results suggest that high doses of melatonin protect against oxidative stress and organ injury [17]. In addition, multiple data support lack of melatonin toxicity even at high doses, and the chances of it producing side effects, if any, are far less than with exogenous antioxidants [17]. Beneficial antioxidant effects were recently shown for melatonin in clinical settings for several chronic diseases [29, 52]. These observations support a melatonin gel formulation having therapeutic potential in patients with oral mucositis. However, systemic administration of melatonin did not achieve effective concentrations in the oral mucosa, so topical application is necessary.

In conclusion, mitochondrial dysfunction is the missing link connecting the NF-κB and NLRP3 pathways of the innate immune response during radiotherapy. Therefore, therapy to prevent mucositis should include a drug that can target the different pathways involved in its development (Fig. 9). The first potential treatment of this type, topical melatonin, has not only the added value of low toxicity and low interference with the irradiation-induced cytotoxic effect [53, 54] but also important oncostatic effects in vitro and in vivo [55–58]. Clinical trials in patients with cancer treated with radiotherapy should assess the clinical efficacy of this new therapy for preventing mucositis.

Acknowledgements

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Conflict of interest

The University of Granada holds a patent on the pharmaceutical composition of melatonin gel and its use in mucositis.

Author contributions

G.E. conceived the study, designed experiments, supervised experiments, and interpreted data. D.A-C. contributed to the design and supervised experiments. D.A-C and G.E wrote the manuscript. F.O. performed the radiation-induced oral mucositis animal treatment and laboratory experiments and interpreted the data. J.C.D. contributed to the radiation-induced mucositis treatment. C.D. performed mitochondrial respiration assay. L.C.L. and C.V. performed CoQ9 and melatonin analyses, respectively. All authors critically reviewed the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Melatonin gel 3% prevents mitochondrial dysfunction induced by irradiation of the tongue. (A) and (B), Quantitative RT-PCR of GPx (A) and GRd (B) in tongues from different groups: control (non-irradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). (C) Quantitative RT-PCR, activity and Western blot analysis, and densitometric quantification of Mn-SOD in the tongue. (D) Activities of the I, II, III, and IV complexes of the electron transport chain in the mitochondria of tongue cells. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

Figure S2. Melatonin gel 3% prevents apoptosis induced by radiation of the tongue through the regulation of apoptotic marker expression. (A) TUNEL assay of tongues

from control (non-irradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). The right panels show details at higher magnification. Scale bars = 50 μm and 20 μm in the left and right panels, respectively. (B) Quantification of the staining in A, $n = 4$ per group. (C) and (D) Quantitative RT-PCR of p53, Bax, Bcl2 (C), and cytochrome c (D) in the tongue, $n = 6$ per group. Data are expressed as mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

Figure S3. Melatonin gel 3% prevents irradiation-induced autophagy and mitochondrial biogenesis marker expression. (A) Western blot analysis and densitometric quantification of Atg12, Beclin-1, and Nix in tongues from control (non-irradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). (B) Western blot and quantitative RT-PCR analysis of NRF1, PGC1-α, TFAM, and sirtuin 1 (Sirt 1) in tongues. The results were quantified by densitometry. $n = 6$ per group. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

Figure S4. Melatonin gel 3% inhibits gene expression in the NF-κB pathway. (A–E) Quantitative RT-PCR of NF-κB (A), IκB-α (B), pro-IL-1β, pro-TNF-α (C), iNOS (D), and COX-2 (E) in tongues from different groups: control (non-irradiated, C) and irradiated rats treated with vehicle (IR), melatonin gel (+aMT 3%), or ip melatonin (+aMT IP). $n = 6$ per group. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus IR.

Figure S5. Effects of melatonin gel 3% in non-irradiated rats. (A) H&E staining of tongue tissue from non-irradiated melatonin gel 3%-treated rats (C + aMT) compared to control (non-irradiated, C) rats, $n = 3$ per group. B, EM of mitochondria from tongue cells. Normal mitochondria were observed in the C and C + aMT groups without mitochondrial damage, $n = 3$ per group. (C) Western blot (WB) analysis and densitometric quantification of OXPHOS in the mitochondria of tongue cells, $n = 6$ per group. (D) GSSG/GSH ratio in the mitochondria of tongue cells, $n = 6$ per group. (E–H) WB analysis and densitometric quantification of P53, Bax, Bcl2, Bax/Bcl2, NLRP3, caspase 1, and NF-κB subunit p65 in nuclear and cytosolic fractions from tongue cells, $n = 6$ per group. I, Quantification of HPLC analyses of melatonin content in tongues from C and C + aMT rats. Data are mean ± S.E.M.

Table S1. PCR primers sequences.