SCIAEON Journal of Radiology

REVIEW ARTICLE

# The Impact of Melatonin and Carbon Ion Irradiation on Mitochondria of Cancer Cells

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## Abstract

**Aim:** Mitochondria are bioenergetic and biosynthetic organelles which provide energy, supply building blocks for new cells, and regulate redox homeostasis, oncogenic signaling, innate immunity and apoptosis. Mitochondria play a crucial role in tumor progression, and targeting mitochondria and mitochondria associated signaling pathways provides therapeutic opportunities to improve the outcome of cancer patients.

**Materials and methods:** A systematic review of the existing literature was conducted using the following search terms: 'melatonin', 'X-ray irradiation', 'charged particle irradiation', 'carbon ion irradiation', 'mitochondria', 'apoptosis' and 'cancer cells'. The search used PubMed and spanned the period from January 2000 to April 2018.

**Results:** The impact of melatonin on mitochondria of cancer cells consisted of 'Melatonin antiproliferative effects require active mitochondrial function in embryonal carcinoma cells', 'Human transporters, PEPT1/2, facilitate melatonin transportation into mitochondria of cancer cells: An implication of the therapeutic potential', 'Melatonin increases human cervical cancer HeLa cell apoptosis induced by cisplatin via inhibition of JNK/Parkin/mitophagy axis' and 'Combination of melatonin and rapamycin therapy for head and neck cancer: Suppression of AKT/mTOR pathway activation, and promotion of mitophagy and apoptosis via mitochondrial function regulation'. The impact of carbon ion irradiation on mitochondria of cancer cells consisted of 'Carbon ion beams induce hepatoma cell death by NADPH oxidase-mediated mitochondrial damage', 'MEK-ERK-dependent multiple caspase activation by mitochondrial proapoptotic Bcl-2 family proteins is essential for heavy ion irradiation-induced glioma cell death', 'Fragmentation level determines mitochondrial damage response and subsequently the fate of cancer cells exposed to carbon ion irradiation' and 'Carbon ion beam triggers both caspase-dependent and caspase-independent pathway of apoptosis in HeLa and status of PARP-1 controls intensity of apoptosis'.

**Conclusion:** Mitochondria are the promising therapeutic targets for cancer treatments in relation to melatonin and carbon ion irradiation. Further preclinical and clinical investigations are indispensable in order to develop innovative anticancer agents.

**Keywords:** melatonin; X-ray irradiation; charged particle irradiation; carbon ion irradiation; mitochondria; apoptosis; cancer cells

## Introduction

Mitochondria are bioenergetic and biosynthetic organelles which provide energy, supply building blocks for new cells, and regulate redox homeostasis, oncogenic signaling, innate immunity and apoptosis [1]. The BCL-2 family of proteins at the mitochondrial outer membrane regulates apoptosis [1]. The release of cytochrome c from the mitochondrial inter-membrane space is inhibited by BCL-2-related anti-apoptotic proteins. Conversely, cytochrome c release is promoted by the BAX and BAK pro-apoptotic proteins [1]. Cytochrome c induces the apoptosome and caspase activation in the cytosol resulting in apoptosis [2-4]. Whether the mitochondrial alteration comprising fusion and fission acts as a cause of tumor progression or a consequence of tumor growth requires further research [5]. Melatonin (N-acetyl-5-methoxytryptamine), an indole synthesized by the pineal gland and possibly by many other tissues [6-8], is a critical inhibitor of cancer initiation, progression and metastasis [9-19]. This substance exerts its anticancer effects in many types of cancer due to its pro-apoptotic, anti-proliferative, anti-cell differentiation and anti-angiogenic actions [20,21]. Many studies have documented melatonin's beneficial effects against oxidative/nitrosative stress, including that involving mitochondrial dysfunction [22-26].

Radiation therapy is an essential modality of cancer treatment. X-rays consist of photons and are commonly used in radiation therapy. X-rays are defined as low LET (Linear Energy Transfer) radiation which produce only occasional ionizations along their trajectories [27]. Failure of radiation therapy using photon irradiation is usually related to metastasis [28]. There is an increasing application of high-LET charged particles such as protons and carbon ions in the treatment of cancer [29-31]. Carbon ions create numerous ionizations along their

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**Received:** Aug 21, 2018; **Accepted:** Aug 27, 2018; **Published:** Aug 29, 2018

trajectories, and induce unrepairable clustered DNA damage. Carbon ion irradiation is more potent in the induction of cytogenetic damage and cytotoxicity of irradiated cells than low-LET X-rays [31,32]. Some recent studies indicate that carbon ion irradiation may be a promising therapeutic modality because of the complex DNA damage, increased apoptosis, and counteractive actions on migration and invasive processes in various types of cancer. This distinguishes it from photon irradiation [33-37]. Increasing evidences indicate that carbon ion irradiation is able to induce mitochondrial damage [38-40].

Understanding of the role of mitochondria in cancer is an innovative approach to targeted therapy. Mitochondria play a crucial role in tumor progression, and targeting mitochondria and mitochondria-associated signaling pathways provides therapeutic opportunities to improve the outcome of cancer patients [41].

## Materials and methods

### Literature search strategy

A systematic review of the existing literature was conducted using the following search terms: 'melatonin', 'X-ray irradiation', 'charged particle irradiation', 'carbon ion irradiation', 'mitochondria', 'apoptosis' and 'cancer cells'. The search used PubMed and spanned the period from January 2000 to April 2018.

### Inclusion and exclusion criteria

We identified reports related to the impact of melatonin and carbon ion irradiation on mitochondria of cancer cells for inclusion. Reports which were published in languages other than English, only published in abstract form, not related to mitochondria of cancer cells, duplicate articles and those containing insufficient detail were excluded. All titles and abstracts were screened to assess whether they were eligible for inclusion. Then abstracts and full texts of all eligible studies were examined and data was evaluated.

## Results

### Literature search results

The search identified 103 potentially eligible articles. After application of the exclusion criteria, only 8 met the criteria and were therefore evaluated. The collected data included the impact of melatonin and carbon ion irradiation on mitochondria of cancer cells. The impact of melatonin on mitochondria of cancer cells consisted of 'Melatonin antiproliferative effects require active mitochondrial function in embryonal carcinoma cells', 'Human transporters, PEPT1/2, facilitate melatonin transportation into mitochondria of cancer cells: An implication of the therapeutic potential', 'Melatonin increases human cervical cancer HeLa cell apoptosis induced by cisplatin via inhibition of JNK/Parkin/mitophagy axis' and 'Combination of melatonin and rapamycin therapy for head and neck cancer: Suppression of AKT/mTOR pathway activation, and promotion of mitophagy and apoptosis via mitochondrial function regulation'. The impact of carbon ion irradiation on mitochondria of cancer cells consisted of 'Carbon ion beams induce hepatoma cell death by NADPH oxidase-mediated mitochondrial damage', 'MEK-ERK-dependent multiple caspase activation by mitochondrial pro-apoptotic Bcl-2 family proteins is essential for heavy ion irradiation-induced glioma cell death', 'Fragmentation level determines mitochondrial damage response and subsequently the fate of cancer cells exposed to carbon ion irradiation' and 'Carbon ion beam triggers both caspase-dependent and caspase-independent pathway of apoptosis in HeLa and status of PARP-1 controls intensity of apoptosis'.

### "The impact of melatonin on tumor stem cells Melatonin antiproliferative effects require active mitochondrial function in embryonal carcinoma cells"

Cancer stem cells are regarded as the energetic force of cancer development and progression. These cells are the principal therapeutic target, since their ability to elude treatments provides a probable explanation for tumor recurrence [42]. Various metabolic features differentiate cancer cells from normal cells [43,44], including a reduced mitochondrial ATP production in an aerobic environment [45,46]. Apart from its renowned functions in circadian and seasonal rhythms [47,48], various actions have been ascribed to melatonin, including the modulation of mitochondrial function [22,23], regulation of cell death [49,50], autophagy [51,52] and an intrinsic antitumoral effect [53]. There is a lack of information related to the functional effects of melatonin on cancer stem cells, especially in reference to its effects on mitochondrial activity [42]. Loureiro and colleagues noticed that P19 cancer stem cells (CSCs) had specific mitochondrial and metabolic features that were changed during cell differentiation. These features were inter-connected with pluripotency and resistance to the mitochondrial agent dichloroacetate [42]. The excitation of mitochondrial activity by culturing P19 stem cells in galactose (glucose-free), glutamine/pyruvate-containing medium decreased their glycolytic phenotype and stemness, activated cell differentiation, and enhanced the vulnerability of P19 cells to dichloroacetate [42]. The P19 cell model provided verification of the effects of melatonin on the same cancer cell line with different degrees of pluripotency, differentiation and mitochondrial activity [42].

### Melatonin decreased P19 cell mass only when oxidative metabolism was used for ATP production

To evaluate the effect of different concentrations of melatonin (0.001, 0.01, 0.1 and 1 mM) on P19 cell four stages of differentiation (Glu-CSCs, Glu-dCCs, Gal-CSCs and Gal-dCCs), cell mass was assessed after 24, 48, 72 and 96 hours of treatment. At 72 hours of incubation, 1 mM melatonin significantly reduced Gal-CSCs and Gal-dCCs cell mass, which was more obvious in Gal-dCCs. The trypan blue dye exclusion assay was performed to ascertain the effects on cell viability after 72 hours of treatment with 0.1 or 1 mM melatonin. Only cells cultured in galactose (glucose-free), glutamine/pyruvate-containing media which depended more on oxidative metabolism for ATP production were vulnerable to 1 mM melatonin [42].

**Melatonin altered mitochondrial membrane potential, oxygen consumption and ATP content in P19 cells**

Melatonin raised mitochondrial membrane potential of all P19 cell groups; achieving significant values with 1 mM melatonin for both groups of CSCs (Glu-CSCs and Gal-CSCs) and with 0.1 mM melatonin for both dCCs groups (Glu-dCCs, Gal-dCCs). No influence on basal oxygen consumption was detected in glycolytic Glu-CSCs treated with melatonin. On the contrary, melatonin reduced basal respiration of more oxidative cells (Glu-dCCs, Gal-CSCs, Gal-dCCs). Even though basal oxygen consumption was not influenced by melatonin in Glu-CSCs, the addition of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) to cells treated with 1 mM melatonin did not increase respiration, indicating that melatonin impaired respiration even in the high glycolytic and resistant Glu-CSCs. Altogether, these results indicated a direct effect of melatonin on the mitochondrial electron transport chain in all groups of P19 cells, particularly in those with an active oxidative metabolism [42].

**Melatonin enhibited pro-oxidant effects, reduced BCL-2 expression and induced a caspase-3-independent cell death in P19 cells with oxidative metabolism**

One mM melatonin significantly enhanced malondialdehyde (MDA) levels, a traditional marker of oxidative stress, in all P19 cell groups compared to their analogous controls. This effect may be associated with the capability of melatonin to reduce the proliferation rate of highly oxidative cells [42].

Since excessive ROS production by mitochondria played a predominant role in mitochondrial outer membrane permeabilization, Loureiro and colleagues evaluated alterations in BCL-2 and BAX protein content. Melatonin-treated cells cultured in galactose media revealed a reduced content of the antiapoptotic protein BCL-2, while this effect was not detected in cells grown in high glucose media. These findings implied that the intrinsic apoptotic pathway had been activated. Untreated Gal-CSCs displayed higher activity of caspase-3 when compared to Glu-CSCs. This may be a result of the forced metabolic remodeling and its related differentiation process induced by the galactose (glucose-free), glutamine/pyruvate-containing medium. The calcein-AM and propidium iodide Live/Dead assay proved that 1 mM melatonin raised the percentage of dead cells (calcein-/propidium iodide+) in cell populations with higher mitochondrial metabolism [42].

Eventually, 0.1 mM melatonin also raised the percentage of dead cells but solely in cells grown in galactose (glucose-free), glutamine/pyruvate-containing medium [42].

**Dichloroacetate enhanced the antiproliferative effect of melatonin**

Co-treatment with 10 mM dichloroacetate and 1 mM melatonin gave rise to reduced Glu-CSCs cell mass. The combination of 10 mM dichloroacetate with 1 mM melatonin exerted a synergistic effect in Gal-CSCs and Gal-dCCs [42].

Loureiro and colleagues estimated the apoptosis-inducing factor (AIF) content in mitochondrial and cytosolic fractions to determine whether AIF released from mitochondria was involved in the antiproliferative effects induced by melatonin. The results documented a higher cytosolic 67-kDa AIF content in Glu-CSCs and in both types of galactose media-grown P19 cells treated with either melatonin alone or in combination with dichloroacetate. An accessory cytosolic band of 57 kDa was detected in melatonin-treated cells grown in galactose medium (Gal-CSCs and Gal-dCCs) and in Glu-dCCs treated with both melatonin and dichloroacetate. This accessory band was probably correlated with a form of AIF which translocated to the nucleus where it activated a caspase-3-independent type of cell death [42].

In brief, highly glycolytic P19 embryonal carcinoma stem cells (CSCs) were more resistant to melatonin antitumor effects whereas cells that depended on oxidative metabolism for ATP production were more sensitive to melatonin. The antiproliferative effect of melatonin was related to reduced oxygen consumption, down-regulation of BCL-2 expression and an enhancement in oxidative stress culminating with caspase-3-independent cell death. The combined treatment of melatonin and dichloroacetate spurred a synergistic effect in cells grown in the galactose medium and gave rise to an inhibitory action in the highly resistant P19 CSCs. Melatonin is a promising agent which utilizes its antiproliferative effect in P19 carcinoma cells through a mitochondria-mediated action. The effect is enhanced by the combination of dichloroacetate when targeting resistant embryonal carcinoma cells.

**“Human transporters, PEPT1/2, facilitate melatonin transportation into mitochondria of cancer cells: an implication of the therapeutic potential”**

The molecular features of melatonin-mediated biological activities have been extensively explored. Nevertheless, the processes of transmembrane transport and the intracellular distribution of melatonin continue to be contentious. Uptake transporters play the role of the cell gatekeepers and aid therapeutic substances to move into target organs [54,55]. The oligopeptide transporters, PEPT1 and PEPT2, account for the absorption and conversation of the digestive products of dietary proteins and peptide-like substances in the intestine and kidneys, respectively [56,57]. The organic anion transporting polypeptide (OATP) and organic anion/cation transporter (OAT/CAT) family are principally located in liver and kidneys, individually. Membrane transporters and metabolic enzymes are two principal factors that control the destiny of the substances moving into cells [58,59]. There is sparse information regarding the membrane transporters of melatonin and its metabolites.

To test the role of PEPT1/2, melatonin transportation was evaluated employing hPEPT1/2-transferred HeLa cells. At each time point, melatonin uptake was notably higher in PEPT1- and PEPT2-HeLa cells compared with mock-Hela cells. Concurrent administration of Bestatin essentially reduce the melatonin uptake in PEPT1- and PRPT2-HeLa cells. Melatonin was carried into PEPT1- and PEPT2-HeLa cells

in a concentration-dependent manner with Km (Michaelis constant) values of 0.6 mM and 1.0 mM, respectively. These results provide firm evidence indicating that melatonin is a substrate for PEPT1/2 [60].

The electrostatic interaction between melatonin and functional site of PEPT1/2 further enhances the stability of the complex, and offers optional conditions for the transfer of molecules via the transmembrane proteins. These results further confirmed that melatonin was the substrate of PEPT1/2. The docking analysis demonstrated that the binding of melatonin to PEPT1/2 was related to their low binding energy and appropriate binding conformation in which melatonin was set in the functional site of PEPT1/2 and suited to the cavity in three-dimensional space [60].

OAT1/3 and OCT2 are the principal transporters that promote the active secretion of endogenous and exogenous substances [61,62]. The uptake of 6OM-S (the sulfated metabolite of 6-hydroxymelatonin) and NAS-S (the sulfate of N-acetylserotonin ) in OAT3-HEK293 cells were enhanced 3.34-fold and 8.88-fold, respectively, compared with the mock-HEK293 cells. The uptake of melatonin, 6-OM (6-hydroxymelatonin) or NAS (N-acetylserotonin) in OAT1/3 and OCT2 transfected HEK293 cells revealed no difference compared to mock cells. Probenecid and verapamil are inhibitors of OAT1/3 and OCT2, individually. The uptake of 6OM-S and NAS-S in OAT3-HEK293 cells was reduced by about 90% in the presence of probenecid [60].

PEPT1/2 is expressed in numerous types of cancer cells. High intracellular melatonin levels have been identified in PC3 and U118 cell lines. A comparison of the uptake rate of melatonin and PEPT1/2 protein expression profiles demonstrated a close association between them. These findings indicate that PEPT1/2 account for the uptake of melatonin by cancer cells [60]. To further examine the crucial role of PEPT1/2 on melatonin uptake, PEPT 1/2- overexpressing PC3 and U118 cells were utilized. Compared with the cells preserved in medium with 10% fetal bovine serum (FBS), the PC3 and U118 cells with 15% FBS significantly enhanced melatonin uptake in a time-dependent manner. The protein levels of PEPT1 and PEPT2 in the PC3 and U118 cells were time-dependently generated by a high concentration of FBS, respectively [60]. These findings revealed that PEPT1/2 promoted uptake of melatonin into cancer cells. Melatonin accumulation in cancer cells was related to the upregulation of PEPT1/2 [60]. Accordingly, PEPT1/2 may be regarded as a melatonin transporter in cells and a potential target for melatonin therapy on cancer.

Flow cytometry was utilized to determine the distribution of cells in their different phases. Melatonin treatment led to elevated fraction of G0/G1 phase in PC3 and U118 cells. G1 arrest coexisted with a reduction in the percentage of cells in the S-phase. Nevetheless, the percentage of cells in the G2/M phase was not changed. Melatonin treatment significantly reduced the protein levels Cyclin D1 and CDK4, fundamental sensors and activators of cell cycle initiation and progression in PC3 and U118 cells in a concentration-dependent manner [60]. These findings revealed that melatonin suppressed the proliferation of PC3 and U118 cells via the suppression of Cyclin D1 and CDK [60].

Melatonin induced apoptosis in different cancer cell lines via the mitochondrial pathway [60]. PEPT1 and PEPT2 were detected both in the cell membrane and in the cytosol. Extensive overlapping of images of the Mito Tracker Red and PEPT1/2 fluorescence signals was detected in the cytoplasm [60]. This finding indicated that the PEPT1 and PEPT2 were also expressed in mitochondria. The mitochondrial fraction was isolated from U118 and PC3 cells. PEPT1 was primarily detected in the mitochondrial fraction of PC3 cells. A very high level of PEPT2 was detected in the mitochondrial fraction of U118 cells. Melatonin concentrations in isolated mitochondria from PC3 and U118 cells were notably suppressed by glycylsarcosine (Gly-Sar). These results further indicate that PEPT1/2 are involved in melatonin uptake by the mitochondria and promote melatonin transport into mitochondria [60].

siRNAs were utilized to knockdown the expression of PEPT1/2 in PC3 and U118 cells with the expression levels of PEPT1 and PEPT2 in these cells being significantly reduced subsequent to siRNAs transfection. Also, the number of mitochondrial PEPT1 and PEPT2 were also notably reduced by the siRNA treatment. The knockdown of PEPT1/2, both in the cellular and mitochondrial levels by siRNA gave rise to the diminished intracellular and mitochondrial melatonin accumulation [60]. The RNA assay presented supplemental evidence to support that PEPT1 and PEPT2 were the functional melatonin transporters of both the cellular membrane and mitochondria in PC3 and U118 cells [60].

Briefly, PEPT1 and PEPT2 transporters undertake a fundamental task in terms of melatonin uptake by cells. Melatonin's membrane transport through PEPT1/2 allows it to exert its inhibitory effect on malignant cells. PEPT1 and PEPT2 were localized in the mitochondrial membrane of human cancer cell lines PC3 and U118. PEPT1and PEPT2 promoted the transportation of melatonin into mitochondria. Melatonin accumulation in mitochondria gave rise to apoptosis of PC3 and U118 cells.

Therefore, PEPT1 and PEPT2 may be a promising cancer cell-targeted melatonin delivery system to promote the therapeutic effects of melatonin in the treatment of cancer.

**"Melatonin increases human cervical cancer HeLa cell apoptosis induced by cisplatin via inhibition of JNK/Parkin/mitophagy axis"**

Cervical cancer is the second-leading cause of cancer death in females, especially in less well developed countries [63,64]. Surgery is carried out for early stage disease, and chemotherapy and/or radiotherapy are delivered based on the pathological findings after surgery. For locally advanced cervical cancer, chemoradiotherapy is suggested as the standard treatment strategy owing to its effectiveness in promoting local control and decreasing distant metastasis [65,66]. Various studies documented that resistance to chemoradiotherapy with

cisplatin-based chemotherapy regimens and radiotherapy leads to poor outcome of the patients with locally advanced cervical cancer [67,68]. To improve survival in these patients, innovative therapeutic approaches involving suppression of drug resistance for the treatment of cervical cancer are necessary [20]. A number of reports claimed that the principal effect of cisplatin-based chemotherapy was the induction of apoptosis of cervical cancer cells [69,70]. Accordingly, the promotion of cisplatin-induced apoptosis may be a major step to enhance the efficiency of chemotherapy.

The target of cisplatin-containing molecules is mitochondria [71]. Cisplatin has effects on mitochondria that result in mitochondrial structure damage [72]. Impaired mitochondria release pro-apoptotic factors into the cytoplasm, resulting in the activation of caspase-9 and caspase-3 [73,74]. This is classified as the caspase-9-dependent cellular apoptosis which accounts for cisplatin-based cellular apoptosis [75]. Mitochondrial quantity and quality is maintained by mitophagy [76,77]. Impaired mitochondria activate Parkin which contributes to the elimination of impaired mitochondria [78,79]. Additionally, Parkin-mediated mitophagy also counteracts the released cytochrome c, giving rise to a blockade of mitochondrial apoptosis [80,81]. Consequently, the control of mitophagic activity is essential to attenuate mitophagy-mediated resistance.

Melatonin may regulate tumor progression and growth through modulation of the life/death balance of cells in breast cancer [82], colorectal cancer [83], lung cancer [84], ovarian cancer [85] and hepatocellular carcinoma [86]. This action would be consistent with the regulatory role of melatonin on cancer cell growth. In addition, a variety of studies document the inhibitory action of melatonin on mitophagy [87,88]. These reports demonstrate that melatonin plays a leading role in the mitophagy inhibition. Chen and colleagues investigated the effect and mechanism of melatonin on HeLa cell apoptosis, especially emphasizing the caspase-9-related apoptosis pathway and mitophagy-mediated anti-apoptotic mechanism [26].

**Melatonin increases caspase-9-mediated mitochondrial apoptosis**

Compared to the normal condition, cisplatin treatment or melatonin applications alone enhance the production of mROS in cancer cells. Thus, the combination of melatonin and cisplatin may further elicit mROS over-production. As a result of mitochondrial injury, the mitochondrial membrane potential ($\Delta\Psi m$) collapses, opening the mitochondrial permeability transition pore (mPTP opening), allowing pro-apoptotic factors to leak into the cytoplasm which are essential elements mediating mitochondrial apoptosis [89,90]. Cisplatin enhanced the mPTP opening in cancer cells while the application of melatonin could further enhanced mPTP opening. Cisplatin gave rise to the release of cyt-c, a pro-apoptotic protein, from mitochondria into cytoplasm and even into the nucleus. In addition, melatonin increased the pro-apoptotic effect of cisplatin. After cyt-c was liberated into cytoplasm, caspase-9, Bad, Bax, and caspase-3 apoptotic proteins were upregulated in the cisplatin treated cells, a response that was further increased when cells were treated with a combination of melatonin and cisplatin [26].

**Melatonin inhibits mitophagy to enhance the sensitivity of HeLa cells to cisplatin**

Recent reports point out that mitophagy, a mitochondrial self-improvement system, is accountable for the cellular resistance to cisplatin [91,92]. Mitophagy was indistinctly activated in HeLa cells treated with cisplatin. The process was significantly inhibited by the application of melatonin. Cisplatin promoted the light chain 3 membrane-bound form II (LC3II) migration to the surface of mitochondria. In contrast, this process was reversed by the treatment with melatonin. Beclin1, Atg5 and p62, markers of mitophagy, were upregulated when HeLa cells were treated with cisplatin while these markers were downregulated under melatonin treatment. The data document that cisplatin triggers mitophagy which is reduced by melatonin. Caspase-9 activity was enhanced in response to melatonin treatment but the activity was reduced with the application of mitophagy activator, carbonilcyanide **p**-triflouromethoxyphenylhydrazone (FCCP). These results demonstrate that mitophagy is initiated as a reaction to cisplatin-mediated mitochondrial damage, and that melatonin is capable of reducing mitophagy [26].

**Melatonin regulates mitophagy via suppressing JNK/Parkin pathways**

Subsequent to mitochondrial damage, Parkin is triggered by a variety of upstream factors such as JNK, ERK and ROS [93,94]. Activated Parkin migrates onto the surface of mitochondria. In addition, Parkin enhances the LC3II accumulation on mitochondria [95,96]. Chen and colleagues found that cisplatin treatment enhanced the expression of p-Parkin. In contrast, melatonin attenuated the levels of p-Parkin on the mitochondrial membrane. Cisplatin treatment activated the JNK pathway as indicated by elevated p-JNK expression. This process was inhibited by melatonin treatment [26]. Activation of JNK by anisomycin not only enhanced the p-JNK expression but also transferred p-Parkin into melatonin-treated HeLa cells. The data indicate that JNK is suppressed by melatonin leading to Parkin activation. Activation of JNK/Parkin decreased the number of transferase-mediated dUTP-biotin nick-end labeling (TUNEL)-positive cells in melatonin-treated cells thereby diminishing the pro-apoptotic effect of melatonin on HeLa cells [26].

These results reveal that JNK/Parkin is activated by cisplatin and avert mitochondrial apoptosis. Conversely, melatonin effectively inhibited the JNK/Parkin and accordingly decreased mitophagy, giving rise to more mitochondrial apoptosis.

In brief, simultaneous stimulation of HeLa cells with cisplatin and melatonin enhanced cellular apoptosis. Additionally, **concurrent** therapy with melatonin and cisplatin significantly increased the mitochondrial structure and function damage, and fundamentally enhanced caspase-9-dependent

mitochondrial apoptosis. Melatonin inactivated mitophagy through interruption of JNK/Parkin pathway, resulting in the inhibition of anti-apoptotic mitophagy. This study validated that melatonin sensitized human cervical cancer HeLa cells to cisplatin-induced apoptosis through inhibition of JNK/Parkin/mitophagy pathways.

**"Combination of melatonin and rapamycin therapy for head and neck cancer: Suppression of AKT/mTOR pathway activation, and promotion of mitophagy and apoptosis via mitochondrial function regulation"**

Squamous cell carcinoma of the head and neck (HNSCC) is the most common malignant neoplasm originating in the upper aerodigestive tract [97-99]. Despite advancements in diagnosis and treatment, the 5-year survival rate for HNSCC has remained comparatively steady at 50% for the past three decades [25]. A significant tendency of local recurrence and locoregional lymph node metastasis is regarded as the crucial prognostic factor of poor clinical outcome [90,91]. At the center of the multiple molecular mechanisms dysregulated in HNSCC, basic, preclinical and clinical findings support the significance of Akt/mTOR signaling pathway in the development of HNSCC [100]. Activation of mTOR and Akt was detected in more than 80% of all HNSCC lesions [100]. Genetic abnormalities in the PI3K/Akt/mTOR pathway are commonly identified in HNSCC [101]. Activation of mTORC1 leads to enhanced protein synthesis and cell survival by direct phosphorylation of its effectors, such as the ribosomal S6K1. S6K1 activation suppresses PI3-kinase signaling, which further reduces the Akt activation [102,103]. Rapamycin is an inhibitor of mTORC1, which gives rise to the suppression of serine/threonine (Ser/Thr) kinase [104]. The PI3K/Akt/mTOR pathway is firmly controlled by multiple feedback loops to adjust growth factor signaling [105]. Suppression of mTOR eliminates feedback inhibition of the pathway, leading to Akt activation which may enhance cancer cell survival, potentially giving rise to more aggressive tumors [106]. Inversion of this feedback loop by rapamycin may reduce its therapeutic effects, whereas a combination therapy with rapamycin and other anticancer agents that eliminate mTOR function and avert Akt activation may enhance antitumor activity [99].

Melatonin (N-acetyl-5-methoxytryptamine) is renowned for its actions in immune system regulation [25]. Moreover, melatonin also promotes downregulation of the phosphorylation of mTOR and Akt in various types of cancer [25]. Based on these features, melatonin is a potential anticancer agent to increase the therapeutic effect of rapamycin. Shen and colleagues have examined the effects of melatonin combined with rapamycin on mitophagy and apoptosis of HNSCC cells by means of mitochondrial function regulation.

**Melatonin inhibits the rapamycin-induced feedback activation of Akt in HNSCC**

Cal 27 cells of human squamous cell carcinoma line were treated with 0, 0.1, 0.5, or 1 mM melatonin and/or 20 nM rapamycin for 48 hours. Rapamycin alone significantly reduced S6 phosphorylation in Cal-27 cells. Nevertheless, ribosomal protein S6 (phospho-S6, p-S6), the downstream target of mTORC1, was significantly decreased in all samples treated with rapamycin and melatonin at all concentrations. Rapamycin reduced the level of Akt

phosphorylation at the Thr site ($p^{T308}$Akt; the mTORC1 phosphorylation site on Akt), in association with the reduction of p-S6. On the contrary, rapamycin elevated Akt phosphorylation at the Ser site ($p^{S473}$Akt; the mTORC2 phosphorylation site on Akt). Feedback activation of mTORC1 through phosphorylation of Akt at serine residue 473 by mTORC2 is regarded as a fundamental limitation of mTORC1-specific inhibitors [107,108]. Nonetheless, treatment with melatonin alone reduced pAkt levels (both S473 and T308), and accordingly reduced the rapamycin-induced enhancement of $p^{S473}$Akt.

Treatment with a combination of rapamycin and melatonin significantly depressed total expression of Akt proteins compared to treatment with rapamycin alone. These results show that rapamycin suppressed mTOR signaling and enhanced the levels of $p^{S473}$Akt. Melatonin reduced the phosphorylation of Akt, consequently suppressing the feedback loop. These findings imply that the modulation of Akt/mTOR signaling may be a pathway by which melatonin acts on HNSCC cancer cells. Additionally, melatonin might increase the anticancer effects of rapamycin [25].

**Melatonin enhances effects of rapamycin, sensitizing cells to treatment**

Shen and colleagues examined the effect of combined treatment with melatonin and rapamycin on the viability of Cal-27 and SCC-9 cells. Morphological alterations after the treatment demonstrated that melatonin administration led to cell detachment and shrinkage. However, trypan blue staining indicated that rapamycin did not exert significant effects on the viability of either cell line. Melatonin reduced cell viability in the presence of rapamycin in a dose-dependent manner, with the maximal effect being achieved at a concentration of 1 mM. Both Cal-27 and SCC-9 cells failed to retain their clonogenic capacity subsequent to the treatment with higher concentrations of melatonin. Following three weeks of treatment, colony numbers were obviously lower subsequent to melatonin administration at all concentrations in Cal-27 cells, especially with melatonin administration at 1 mM in SCC-9 cells. The clonogenic assay proved that melatonin gave rise to reproductive death of cells. These findings revealed that there was no noticeable anti-clonogenic capacity of rapamycin in HNSCC Cal-27 and SCC-9 cells. On the contrary, the combination of melatonin and rapamycin exerted powerful dose-dependent anti-proliferative effects on both HNSCC cell lines.

Combined treatment with rapamycin and melatonin at concentrations of 0.5 and 1 mM elevated expression of the pro-apoptotic protein Bax, reduced expression of the anti-apoptotic protein Bcl2, and increased the Bax/Bcl-2 ratio.

Only high melatonin concentrations (0.5 and 1 mM) elevated the anticancer effect of rapamycin, resulting in apoptosis of HNSCC cell lines [25].

The findings indicated that both Cal-27 and SCC-9 cell lines displayed in vitro resistance to rapamycin. In addition, combining rapamycin and melatonin significantly reduced cell viability and colony number, and enhanced apoptosis in a dose-dependent manner. These results document that rapamycin induces Akt activity, whereas melatonin inhibits its induction. In particular, inhibition of mTOR in HNSCC cells reduced the expressions of essential molecules involved in cellular metabolism [25].

**Melatonin directly controls mitochondrial function, enhancing the biological effects of rapamycin**

Shen and colleagues also estimated mitochondrial respiration capacity in Cal-27 and SCC-9 cells, and observed that treatment with rapamycin alone led to a reduced basal oxygen consumption rate (OCR) in both Cal-27 and SCC-9 cells. To evaluate the electron transport chain integrity, oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and a combination of rotenone and antimycin were added to both cell lines. After addition of oligomycin, an ATP synthase inhibitor, both cell lines exhibited reduced oxygen consumption while maintaining detectable respiration. This finding indicated that the occurrence of ATP synthesis was associated with oxygen consumption. These cells were subsequently treated with FCCP to uncouple respiration, followed by treatment with a combination of rotenone and antimycin. The addition of FCCP led to enhancement of oxygen consumption to the maximal respiratory capacity (ETC). The addition of rotenone and antimycin eliminated mitochondrial respiration [25].

The data revealed that rapamycin reduced basal respiration, the ETC, ATP turnover and proton leakage. Collectively, these findings indicate that rapamycin-treated cells display a lower capacity for oxidative phosphorylation. In addition, rapamycin reduces extracellular acidification rate (ECAR) compared to the basal control.

These results demonstrated that rapamycin may reduce metabolic rate in HNSCC. Combined treatment with both rapamycin and melatonin induced a prolonged reduction in oxygen consumption, and a noticeable drop in proton leakage. Additionally, melatonin dose-dependently raised ECAR values. Both cell lines demonstrated enhanced proton leakage with the combination of melatonin and rapamycin in contrast to rapamycin alone. Compared to in SCC-9 cells, Cal-27 cells exhibited a greater difference in proton leakage between the groups treated with and without melatonin. These results were compatible with considerable resistance of SCC-9 to the combined treatment.

The data concerning respiration were in agreement with the assessment that melatonin increased the effects of rapamycin, regarding the reduction in the number of mitochondria. Analysis of OXPHOS demonstrated that treatment with melatonin alone resulted in significant elevation in the expressions of respiratory complexes I, III, and IV. Combined treatment enhanced the levels of complexes I and III which was in contrast to the control or treatment with rapamycin alone, with the strongest effects detected at the highest melatonin concentrations (0.5 and 1 mM). The combination of rapamycin and melatonin remarkably increased mitochondrial mass in contrast to the treatment with rapamycin alone. The mtDNA/nDNA ratio revealed that combined treatment with rapamycin and 1 mM melatonin notably elevated mtDNA. In general, these results suggest that melatonin enhances the anticancer effect of rapamycin by increasing the number of dysfunctional mitochondria [25].

**Melatonin affects redox homeostasis in head and neck cancer cells**

Complex I and complex III of the ETC are principal sites of ROS production. Shen and colleagues reported that combined treatment with melatonin and rapamycin remarkably enhanced the expression of complex I and complex III. Combined treatment with rapamycin and melatonin significantly and dose-dependently enhanced ROS generation in contrast to rapamycin alone. Nevertheless, the rise in ROS generation by the combination of melatonin and rapamycin was eliminated by the administration of 1 mM melatonin. Analysis of lipoperoxide (LPO) levels and glutathione disulfide (GSSG)/glutathione (GSH) ratio also confirmed that oxidative stress was enhanced by treatment with rapamycin and 0.1 or 0.5 mM melatonin. Oxidative stress was decreased following the treatment with 1 mM melatonin only. Melatonin also dose-dependently inhibited the activity of the antioxidant enzyme with the greatest effect being observed at 1 mM. These experimental findings favor the likelihood that melatonin increases the effects of rapamycin via inhibition of Akt pathways and induction of oxidative stress [25].

**Effect of combined treatment with melatonin and rapamycin in head and neck squamous cell carcinoma xenografts**

Athymic nude mice were implanted with Cal-27 cells. Tumor-bearing mice were arbitrarily divided into three groups: untreated control group, rapamycin treatment, and treatment with both rapamycin and melatonin. Both rapamycin treatment and combined treatment groups gave rise to significant decrease of tumor volume contrasted with the control group. H&E staining identified numerous mitotic cells in the control group, whereas both treated groups had tumor cells that manifested signs of apoptosis, characterized by cellular volume reduction, nuclear fragmentation (apoptotic bodies), and chromatin condensation. Nevertheless, the group treated with both melatonin and rapamycin demonstrated a greater accumulation of apoptotic bodies and tumor cell necrosis [19].

In brief, these results implied that combined treatment with rapamycin and melatonin synergized such that the anticancer effects on HNSCC cells were increased. The findings may provide the potential for further clinical assessment of

melatonin as an adjuvant agent with rapamycin to minimize local recurrence and locoregional lymph node metastasis. Accordingly, there may be a promising outlook for improving the survival of HNSCC patients.

**The impact of carbon ion irradiation on tumor stem cells**

**Carbon ion irradiation induces hepatoma cell death by NADPH oxidase-mediated mitochondrial damage**: It is crucial to investigate the cytotoxic effects of carbon ion irradiation on human carcinoma cell line as it related to mitochondrial damage, the molecular process of NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) activation, and the correlation between NADPH oxidase and mitochondria. Mitochondria expend approximately 90% of the oxygen utilized by cells [38]. The principal biological function of mitochondria is to execute energy-producing oxidative phosphorylation that synthesizes ATP to meet the demands of cellular functions [109]. Mitochondria are the fundamental source of reactive oxygen species (ROS) [110]. Mitochondrial dysfunction is specified by high ROS generation and disruption of the mitochondrial membrane potential, and is often related to mtDNA damage [111]. Gradual accumulation of mutations caused by oxidative stress may lead to mitochondrial dysfunction [38]. Dysfunctional mitochondria bring about more ROS, initiating a feed-forward loop in which ROS-mediated oxidative damage to mitochondria gives rise to even more ROS production [112]. Photon irradiation might facilitate ROS formation, leading to apoptosis of tumor cells [38].

NADPH oxidase was initially recognized as a principal source of ROS. The catalytic hub of this oxidase is the integral membrane protein gp91phox (known as Nox2). Various studies disclosed that angiotensin II (AngII) enhanced production of mitochondrial ROS, reduced mitochondrial membrane potential, and gave rise to mitochondrial dysfunction [113]. Apocynin (APO) notably reduces mitochondrial ROS generation in reaction to AngII [38]. Promotion of mitochondrial ROS generation involves the total enzymatic activity of NADPH oxidase. NADPH oxidase activity can be elicited under various conditions such as ultraviolet radiation [114] and X-rays [115].

**Carbon ion radiation induced NADPH oxidase activation by means of $p47^{phox}$ translocation**

Sun and colleagues employed immunofluorescence staining of membrane proteins and flow cytometry to detect the unique accumulation of $p47^{phox}$ on the membrane. NADPH oxidase activity was elevated significantly 30 minutes after 4 Gy of carbon ion irradiation for HepG2 cells. Carbon ion radiation elicited NADPH oxidase activation by $p47^{phox}$ translocation. Treatment with 100 μM APO 30 minutes before 4 Gy of carbon ion irradiation might interrupt $p47^{phox}$ translocation and suppress NADPH oxidase activation [38].

**The mitochondrial vicious circle was initiated by carbon ion radiation induced NADPH oxidase activation**

The level of mtDNA damage and ROS in HepG2 cells increased markedly 30 minutes after carbon ion irradiation. Treatment with 100 μM APO 30 minutes in advance of carbon ion irradiation successfully obviated mtDNA damage and the marked rise in ROS. Similar effects were not detected when APO was given 30 minutes after carbon ion irradiation. The data revealed that NADPH oxidase activation was an initiator of mitochondrial damage. As soon as mitochondria enter a vicious circle, abundant ROS originated in the mitochondria. Treatment with the mitochondria-targeted antioxidant MitoQ at 1 μM 30 minutes after carbon ion irradiation effectively eliminated ROS and alleviated mtDNA damage [38].

**Carbon ion radiation increased mitochondrial superoxide generation and enhanced mtDNA oxidative damage**

HepG2 cells irradiated with carbon ion beams gave rise to enhanced mitochondrial superoxide generation compared to those levels in untreated controls. The damaged DNA product, 8-OHdG, levels were higher in mitochondria than that in the nucleus. Accordingly, mitochondria were the principal source of ROS after carbon ion irradiation in HepG2 cells. Mitochondrial ROS and mtDNA oxidative damage were both reduced by administration with 100 μM APO 30 minutes before carbon ion irradiation or treatment with 1 μM MitoQ 30 minutes after radiation exposure [38].

**Carbon ion radiation caused the collapse of mitochondrial membrane potential**

HepG2 cells irradiated with 4 Gy of carbon ion beams displayed a noticeable reduction in mitochondrial function and an increase in cytoplasmic staining, indicating a loss of mitochondrial membrane potential. Sun and colleagues observed that treatment with 100 μM APO 30 minutes before carbon ion irradiation preserved mitochondrial membrane potential. On the contrary, this effect was not apparent after treatment with APO 30 minutes after carbon ion irradiation [38].

**Carbon ion radiation induced HepG2 cell death and inhibited proliferation**

HepG2 cells exposed to 4 Gy of carbon ion irradiation showed an increased proportion of apoptotic cells compared to the control group 24 hours after irradiation. HepG2 cells irradiated with 4 Gy of carbon ion beams also exhibited a significant reduction in cell number. The cells exposed to 4 Gy of carbon ion irradiation also exhibited a substantial inhibition of cell growth compared to the control group. Treatment with 100 μM APO 30 minutes before irradiation or 1 μM MitoQ 30 minutes after irradiation led to a reduction in the killing effect and in proliferation inhibition. Conversely, these effects were not detected when HepG2 cells were treated with APO 30 minutes after carbon ion irradiation. The data indicated that NADPH oxidase activation played the role of the initiator of cell death-induced mitochondrial damage [38].

Sun and colleagues also noticed that carbon ion irradiation impaired mtDNA, increasing ROS levels and inducing HepG2 cell apoptosis. After carbon ion irradiation, the principal sources of ROS and the main causes of critical oxidative

DNA damage were mitochondria. Comet assays and colony formation assay demonstrated that ρ+ (wild type HepG2) cells were radiosensitive. The high degree of radiation resistance detected in ρ0 cells indicated that cytotoxicity induced by carbon ion irradiation was dependent on mitochondria. This finding shows that there is a direct relationship between the cytotoxic effect of carbon ion radiation on cancer cells and the mitochondrial vicious circle [38].

Briefly, mitochondria accounted for an early onset of ROS formation with subsequent minor but a prompt oxidative stress situation based on activation of NADPH oxidase. NADPH oxidase activation was an initiator of the damage to the mitochondria. Enhancement of NADPH oxidase activation, mitochondrial damage and ROS levels was found after 4 Gy of carbon ion irradiation of HepG2 cells. The disruption of mitochondrial membrane potential, oxidative stress and radiation induced mtDNA damage were effectively prevented when HepG2 cells were treated with APO before carbon ion irradiation. A lack of protective effects was found when HepG2 cells were treated with APO after carbon ion irradiation. The mitochondria-targeted antioxidant MitoQ disrupted the vicious circle resulting in the avoidance of HepG2 cell apoptosis.

**MEK–ERK-dependent multiple caspase activation by mitochondrial proapoptotic Bcl-2 family proteins is essential for heavy ion irradiation-induced glioma cell death**

It is essential to explore the detailed molecular mechanism of carbon ion irradiation-induced glioma cell death, including the mode of cell death, the involvement of crucial intracellular cell death signal cascades and carbon ion irradiation-specific cell death signal transducers, in order to improve the efficacy of carbon ion irradiation for the treatment of glioma.

**Dose-dependent carbon ion irradiation induced cell death of human glioma cell lines with apoptotic features**

Tomiyama and colleagues irradiated T98G and U251 human glioma cell lines with carbon ion beams in a dose range of 1-10 Gy, which was compatible with the clinical application of carbon ion irradiation for the treatment of glioma. Induction of cell death in both cell lines was proven to be dose-dependent at 48 hours after irradiation. To detect apoptosis, further staining of nuclei utilizing Hoechst 33342 reagent was performed. Cells with apoptotic nuclear morphology showed dose-dependent radiation-mediated changes in both T98G and U251 glioma cell lines. This finding indicated that carbon ion irradiation triggered apoptotic cell death in human glioma cells [39].

**Multiple caspases were involved in carbon ion irradiation-induced cell death**

Caspase 9, caspase 3 and caspase 8 were triggered upon the induction of glioma cell death by carbon ion irradiation. Pan-caspase inhibitors efficiently attenuated the caspase activation, processing of poly-ADP ribose polymerase (PARP), apoptosis and cell death of T98G and U251. By comparison, each specific caspase inhibitor interrupted carbon ion irradiation-induced glioma cell death fairly efficiently but not as much as pan-caspase inhibitors [39]. These results indicate that caspases are functionally fundamental for carbon ion irradiation-induced T98G and U251 glioma cell death.

**Bcl-2 family proteins regulated carbon ion irradiation-induced caspase activation and apoptosis of glioma cells at the mitochondrial level**

Pro-apoptotic Bcl-2 family proteins, particularly BCL-2-associated X protein (Bax) and BCL-2- associated killer (Bak), played a critical role in cell death activated by various stimuli with the involvement of mitochondria [39]. Activation of Bax and Bak was essential for mitochondrial outer membrane permeabilization and transduction of the cell death signal by these organelles. Upon activation, Bax translocated from the cytosol to the mitochondrial outer membrane and gave rise to a self-oligomer; Bak also gave rise to a pore-forming oligomer in the mitochondrial outer membrane [39]. After carbon ion irradiation, Bax translocation from the cytosol to the mitochondria was detected, and self-oligomerization of both Bax and Bak was identified [39]. Carbon ion irradiation-induced glioma cell death was effectively inhibited not only by Bcl-2 or Bcl-xl overexpression but also by the double knockdown of Bax and Bak, while single knockdown of Bax or Bak resulted in partial inhibition. These findings document that both Bax and Bak are crucial for carbon ion irradiation-induced glioma cell death and that caspases are activated downstream of the mitochondrial pro-apoptotic Bcl-2 family protein. Carbon ion irradiation-induced oligomerization of Bax was not influenced by either pan-caspase or specific caspase inhibitors in T98G cells, while pan-caspase inhibitors suppressed Bax oligomerization in U251 cells [39].

**The MEK–ERK cascade positively regulated carbon ion irradiation-induced cell death**

Knockdown of p38alpha or MEK1/2 especially down-regulated the expression of their individual targets and also inhibited the activation of p38 MAPK and ERK1/2, respectively. T98G and U251 cells persistently expressing DN (dominant-negative) ERK2 successfully reduced carbon ion irradiation-induced cell death compared with non-transfected cells or cells stably transfected with the empty vector. Thus, ERK activity is indispensable for glioma cell death. MEK1/2 knockdown attenuated caspases activation, Bax and Bak stimulation, and cytochrome c release 48 hours after carbon ion irradiation in both cell lines [39]. These results suggest that the MEK-ERK pathway regulates carbon ion irradiation-induced glioma cell death upstream of the mitochondria.

**Recovery of ERK activation within 36 hours, but not thereafter, was essential for carbon ion irradiation-induced cell death**

MEK-specific inhibitors interrupted the carbon ion irradiation-induced glioma cell death signal upstream of the mitochondria and, accordingly, cell death itself. Carbon ion irradiation-induced Bax oligomerization, cytochrome c release, caspase-3 activation, and cell death were successfully inhibited by one protocol (selective inhibition of ERK activity 2-36 hours after

irradiation) and minimally by the other protocol (selective inhibition of ERK activity later than 36 hours after irradiation) in both glioma cell lines [39]. The findings confirm that the recovery of ERK activation during the first 36 hours after carbon ion irradiation showing that this period is critical for carbon ion irradiation-induced glioma cell death [39].

**Stimulation of the ERK cascade enhanced carbon ion irradiation-induced glioma cell death**

Carbon ion-irradiated glioma cells pretreated with EGF, an ERK cascade initiator, revealed a sustained activation of ERK compared with carbon ion treatment alone. Cell death, Bax oligomerization, and caspase-3 activation were significantly increased by EGF pretreatment. The findings show that stimulation of the ERK cascade enhances carbon ion irradiation-induced glioma cell death [39].

**Limited dependence of X-ray-induced glioma cell death on the MEK–ERK pathway**

The activation of ERK by X-ray irradiation was altered in both T98G and U251 cells, but the alteration was different from that caused by carbon ion irradiation. When X-ray-irradiated T98G/U251 cells were simultaneously treated with MEK inhibitors, cell death was not notably impacted in either glioma cell line; this was different from the changes seen after carbon ion irradiation. In addition, T98G and U251 cells stably expressing DN ERK2 did not exhibit **meaningfully** reduced caspase-3 activation and cell death after X-ray irradiation compared with control cells. Thus, the MEK-ERK cascade may have an essential role in carbon ion irradiation-induced glioma cell death but not in X-ray-induced glioma cell death.

Tomiyama and colleagues demonstrated that carbon ion irradiation-mediated glioma cell death, particularly within 48 hours after irradiation, led to apoptosis [39]. Many tumor cells treated with conventional irradiation undergo apoptosis [116]. Histological examination of human tumor specimens revealed that high-dose conventional irradiation resulted in tumor cell necrosis [117]. Within several days after carbon ion irradiation, necrosis did not occur in glioma cells. The principal features of carbon ion irradiation-induced glioma cell death were compatible with apoptosis [39].

The reactivation of ERK observed 2-48 hours after carbon ion irradiation appeared to play a critical role in carbon ion irradiation-induced glioma cell death. In addition, even the comprehensive inhibition of ERK by U0126 did not prevent cell death, while pan-caspase inhibitors suppressed carbon ion irradiation-induced glioma cell death nearly totally [39]. The findings indicate that an ERK-independent pathway probably gives rise to the death of cells after exposure to carbon ion irradiation.

In brief, carbon ion irradiation elicits reactivation of the MEK-ERK pathway following its initial downregulation, which then induces the activation of the mitochondrial caspase cascade, culminating in apoptotic death of glioma cells. The essential molecules in this cell death signaling pathway are potential targets for enhancement of the effect of carbon ion therapy against gliomas.

**"Fragmentation level determines mitochondrial damage response and the fate of cancer cells exposed to carbon ion irradiation"**

Ionizing radiation promotes mitochondrial damage [118], involving oxidative phosphorylation (OXPHOS) activity and oxygen consumption [119], alterations in copy number and super-coiling [120], and oxidative damage to mtDNA [121].

Targeted cytoplasmic microbeam irradiation led to mitochondrial fragmentation and a decline of cytochrome c oxidase and succinate dehydrogenase activities [122]. Long-term fractionated irradiation increased mitochondrial membrane potential and cytochrome c oxidase activity in neural progenitor stem cells [123,124]. The findings support the idea that radiation exposure interrupts the mitochondrial biogenesis, resulting in mitochondrial dysfunction. Mitochondrial fragmentation enhanced Bax-dependent cytochrome c redistribution from mitochondria to the cytoplasm, a process that initiates activation of caspase protease executioners [125].

In the study of Jin and colleagues, the human cervical cancer cell line HeLa and human breast cancer cell lines MCF-7 and MDA-MB-231 were irradiated with carbon ion beam at low dose of 0.5 Gy and high dose of 3 Gy, respectively. The study demonstrated that carbon ion irradiation induced two distinct mitochondrial morphological alterations and associated responses in these cancer cells. Cells exposed to carbon ion irradiation of 0.5 Gy exhibited modest fragmentation of mitochondria. These impaired mitochondria could be removed by mitophagy. On the contrary, mitochondria damaged by 3 Gy irradiation divided into punctate and clustered fragments, which were related subsequently to apoptotic cell death [40].

These results show that the degree of fragmentation determined mitochondrial damage responses and documents a stress response model that describes the distinct responses to mitochondrial damage caused by carbon ion irradiation at different doses in cancer cells.

**"Carbon ion beam triggers both caspase-dependent and caspase-independent pathways of apoptosis in HeLa cells with the status of PARP-1 controlling the intensity of apoptosis"**

To distinguish and quantify modes of cell death and uncover the mechanism are essential in recognizing biological effects of high LET radiation. Poly (ADP-ribose) polymerase-1 (PARP-1) plays a vital role in DNA repair and its inhibitors are recognized as radiosensitizers for low LET radiation [126]. Ghorai and colleagues investigated the mechanism of apoptosis induced by carbon ion irradiation (CII) and the role of PARP-1 in CII-induced apoptosis [127].

PARP-1 knocked down HeLa cells (labelled as HsiI) and PARP-1 knocked down HEK 293T cells (labelled as HEKsiI) were produced by stably transfecting HeLa and HEK 293T cells with siRNA insert of PARP-1 containing plasmid pRNA-U6.1 using Lipofectamine$^{TM}$2000. HeLa cells were

transfected with vector plasmid pRNA-U6.1 without siRNA insert of PARP-1 and labelled as H-vector. HEK 293T cells were processed in the same way and labelled as HEK-vector [127].

Sixty-six per cent suppression of PARP-1 protein expression was noticed in HsiI cells in comparison with H-vector [127]. The extent of DNA damage was estimated by tail moment, %tail DNA and %head DNA. Higher DNA damage demonstrated longer tail and shorter head resulting in greater tail moment, greater area of tail or greater %tail DNA and smaller head area or smaller %head DNA. Tail moment in HsiI cells was significantly higher than H-vector at 4 Gy of CII. Greater %tail DNA and smaller %head DNA were observed in HsiI cells compared with H-vector at all doses of CII. The data show that CII induces DNA breaks in both H-vector and HsiI cells but the quantity of DNA breaks is greater in HsiI cells compared with H-vector [127].

Nucleosomal ladder formation is a distinctive feature of apoptosis. The intensity of nucleosomal ladder is greater in HsiI cells than H-vector at the corresponding doses (0-4 Gy). This suggests that apoptosis generated by CII is higher in HsiI cells. Apoptotic body formation is significantly more obvious in HsiI cells compared with H-vector at and above 2 Gy of CII. The greater number of apoptotic cells in un-irradiated HsiI (6.33 %) than those in un-irradiated H-vector (4.28 %) indicated that apoptosis was elicited by the decline of PARP-1. Significantly higher apoptosis was detected in HsiI cells compared with HeLa cells at all doses [127].

Overall caspase-3 activity at all doses was higher in HsiI cells compared with H-vector. Caspase-3 activity in un-irradiated HsiI cells was significantly elevated above that in un-irradiated H-vector. Activation of caspase-9 at all doses was significantly higher in HsiI cells compared with H-vector. Caspase-9 activity in un-irradiated HsiI cells was verifiably higher than that of un-irradiated H-vector. The results illustrated that caspase-9 was triggered by knocking down the PARP-1 gene and it initiated caspase-3 thereafter [127].

The average value of the fluorescence intensity of rhodamine 123 in healthy un-irradiated H-vector was taken as 100 % MMP (mitochondrial membrane potential). The decline of the fluorescence intensity was significantly greater for HsiI cells at all doses of CII. This indicated that CII induced a greater decline of MMP in HsiI cells compared with that in H-vector. A 37% decline in MMP was detected upon knocking down of the PARP-1 gene in un-irradiated HsiI cells. Furthermore, Ghorai and colleagues confirmed this result in HeLa and HEK 293T cell lines using fluorescence-activated cell sorting (FACS). This result confirmed that knocking down of PARP-1 gene led to the loss of MMP which was not related to cell type [127]. Knocking down the PARP-1 gene initiated the intrinsic pathway of apoptosis through loss of MMP that elicited activation of caspase-9 followed by activation of caspase-3 in un-irradiated HsiI cells [127].

The extrinsic pathway of apoptosis was examined by measuring caspase-8 activation using fluorescence spectrophotometry. The result revealed a dose-dependent enhancement of caspase-8 activation after CII in both H-vector and HsiI cells. However, caspase-8 activity was significantly higher in HsiI cells compared with H-vector at and above 1 Gy. The expression of p53 was examined using an immunofluorescence technique in both H-vector and HsiI cells irradiated with CII. There was a steady rise of p53 expression in PARP-1 knocked down cells after CII and a significant rise was detected at 2 Gy and 4 Gy compared with un-irradiated controls [127].

Briefly, the reduction of PARP-1 expression activated apoptotic signals through intrinsic and extrinsic pathways in un-irradiated cells. Carbon ion irradiation further enhanced both intrinsic and extrinsic pathways of apoptosis along with upregulation of p53 in HsiI cells leading to a greater degree of apoptosis in HsiI than H-vector.

## Discussion

Mitochondria are the major organelles in studies of cancer biology because of their imperative roles in cancer initiation, growth, survival, recurrence, metastasis and resistance to drug or radiation [44]. The definitive means by which cancer cells retain an anaerobic metabolism in the presence of oxygen and the connection between carcinogenesis and stem cell metabolism are not entirely known [128]. The anti-tumor effect of melatonin is related to an arrest at S-phase, a reduction of the mitochondrial electron transport chain (ETC), generation of reactive oxygen species, BCL-2 down-regulation and AIF release. C**onsequently**, combination therapy with melatonin and the activation of mitochondrial metabolism represents promising strategies reducing cancer stem cell resistance [44].

In a study by Huo and colleagues, the cellular transporters of PEPT1/2 and OAT3 were identified to enhance the transmembrane transportation of melatonin and its sulfation metabolites [60]. Melatonin has been utilized to treat several human tumors.

Cancer cell lines derived from prostatic carcinoma, glioma, and pancreatic cancer display high levels of PEPT1 or PEPT2 expression [60]. This finding indicated that the therapeutic effects of melatonin on these tumors may be related to the PEPT1/2 transporters. The data revealed a positive correlation between the intracellular melatonin levels and the expression of PEPT1/2 in eight cancer cell lines [60]. Functional studies confirmed that PEPT1/2 in mitochondria vigorously transported melatonin into mitochondria. Additionally, knockdown of PEPT1/2 reduced the mitochondria-dependent apoptosis induced by melatonin [60]. Thus, mitochondrial PEPT1/2 are also related to the inhibitory effect of melatonin on these cancer cells. PEPT1/2 could be utilized as a cancer cell-targeted melatonin delivery system for cancer therapy.

Chen and colleagues demonstrated that melatonin was capable of increasing human cervical cancer HeLa cells apoptosis induced by cisplatin [20]. This study illustrated the important feature of melatonin as an adjuvant to enhance the efficiency of cisplatin involving mitochondrial apoptosis, mitochondrial dysfunction, mitophagy and JNK/Parkin pathways. Melatonin

may also be a potential adjuvant for the treatment of cervical cancer [26]. Further in depth exploration **of this subject** should be performed to provide adequate evidence for clinical application [26].

Rapamycin is an inhibitor of the mTOR pathway and displays an intense anticancer phenotype in both preclinical and clinical trials [25]. Shen and co-workers reported that melatonin activated mitophagic and apoptotic processes through regulation of mitochondrial function in HNSCC. The result of this study indicated that the combination of melatonin and rapamycin impacted mitochondrial homoeostasis in the HNSCC cells lines. Their study demonstrated a substantial elevation in intracellular ROS levels following combined treatment with melatonin and rapamycin compared to treatment with rapamycin alone [25]. The study of Shen and colleagues illustrated a connection between melatonin-induced mitophagy and apoptosis in HNSCC, which could give rise to the development of novel strategies for cancer therapy.

Sun and co-workers investigated a probable mechanism by which carbon ion irradiation brought about cancer cell death involving mitochondrial damage induced by NADPH oxidase [38]. After carbon ion irradiation, the principal sources of ROS and the crucial site of serious oxidative DNA damage were mitochondria. Increased ROS enhanced oxidative stress, elevated mtDNA damage, **eventually** culminated in cell death [38]. HepG2 cells which were treated with 4 Gy of carbon ion irradiation exhibited enhancement of NADPH oxidase activation, mitochondrial damage and ROS levels. Carbon ion irradiation impaired mtDNA, elevated ROS levels and caused HepG2 cell death [38].

A principal feature of carbon ion irradiation-induced glioma cell death was consistent with a higher degree of apoptosis [39]. Tomiyama and colleagues reported that the reactivation of ERK observed 2-48 hours after carbon ion irradiation likely played a crucial role in carbon ion irradiation-induced glioma cell death [39]. The near total inhibition of ERK by U0126 did not avert cell death totally, while pan-caspase inhibitors suppressed carbon ion irradiation-induced glioma cell death by essentially 100% [39]. Consequently, these results indicated that an ERK-independent pathway could gave rise to carbon ion irradiation-induced glioma cell death.

Low-dose carbon ion irradiation elicited mitophagy in cancer cells [40]. When the radiation dose was increased, release of cytochrome c **was followed by** apoptosis which was dominant. Jin and colleagues reported that carbon ion irradiation induced moderate fission of mitochondria at a low dose whereas high-dose carbon ion irradiation primarily generated serious fragmentation of mitochondria [40]. This result is similar to that reported by Suzuki-Karasiki and co-workers [129]. Further investigation is mandatory to elucidate the mitochondrial dynamics and response of normal cells to carbon ion irradiation. It might improve the efficacy of carbon ion irradiation for the treatment of cancer.

Okamoto and colleagues reported that photon irradiation gave rise to apoptosis followed by apoptosome formation and then activation of caspase-3 [130]. Photon irradiation also generated apoptosis through caspase-8 activation [131]. Nucleosomal ladder formation was the distinctive feature of apoptosis in this case. The intensity of nucleosomal ladder was higher in HsiI cells than H-vector at correlated doses (0-4 Gy) of carbon ion irradiation [127]. The decline of the fluorescence intensity of rhodamine 123 was significantly greater for HsiI cells at all doses of carbon ion irradiation [127]. Knocking down of PARP-1 gene initiated the intrinsic pathway of apoptosis through loss of MMP that elicited activation of caspase-9 followed by activation of caspase-3 in un-irradiated HsiI cells. Dose-dependent enhancement of caspase-8 activation was noted after carbon ion irradiation in both H-vector and HsiI cells [127]. It seems clear that mitochondria are promising therapeutic targets for cancer treatments in relation to melatonin and carbon ion irradiation.

## Conclusion

Highly glycolytic P19 embryonal carcinoma stem cells (CSCs) are resistant to melatonin's antitumor effects. The combined treatment of melatonin and dichloroacetate caused a synergistic effect in cells grown in the galactose medium and gave rise to a suppressive action in the highly resistant P19 CSCs. Melatonin is thus a likely beneficial agent which utilizes its antiproliferative effect to inhibit P19 embryonal carcinoma cells through a mitochondria-mediated action.

PEPT1 and PEPT2 transporters may play a crucial role in melatonin uptake in cells. Melatonin's membrane transportation through PEPT1/2 may related to inhibitory effect on malignant cells. PEPT1and PEPT2 promote the transportation of melatonin into mitochondria. Melatonin accumulation in mitochondria give rise to apoptosis of PC3 and U118 cells. Therefore, PEPT1 and PEPT2 may be employed as a cancer cell-targeted melatonin delivery system to advance the therapeutic effects of melatonin in cancer treatment.

Simultaneous stimulation of HeLa cells with cisplatin and melatonin enhanced cellular apoptosis greater than that induced by either agent alone. Additionally, **concurrent** therapy with melatonin and cisplatin significantly elevated the damage to mitochondrial structure and function, and fundamentally enhanced the caspase-9-dependent mitochondrial apoptosis. Melatonin inactivated mitophagy through interruption of JNK/Parkin pathway. These results validated that melatonin sensitized human cervical cancer HeLa cells to cisplatin-induced apoptosis through inhibition of JNK/Parkin/mitophagy pathways.

Shen and colleagues indicated that combined treatment with rapamycin and melatonin increased the anticancer effects of rapamycin in HNSCC cells. This finding may provide the potential for further clinical assessment of melatonin as an adjuvant agent with rapamycin to minimize local recurrence and locoregional lymph node metastasis. Accordingly, this would provide a promising outlook for improving the survival of HNSCC patients.

Enhancement of NADPH oxidase activation, mitochondrial damage and ROS levels was found after 4 Gy of carbon ion irradiation of HepG2 cells. The disruption of mitochondrial membrane potential, oxidative stress and radiation induced mtDNA damage were effectively prevented when HepG2 cells were treated with APO before irradiation.

Carbon ion irradiation gave rise to reactivation of the MEK-ERK pathway following its initial downregulation, which then initiated the activation of the mitochondrial caspase cascade, reaching a peak in apoptotic death of glioma cells. These molecules necessitated in this cell death signaling pathway could be potential targets for enhancement of the effect of carbon ion therapy against gliomas.

Cancer cells exposed to carbon ion irradiation of 0.5 Gy manifested modest fragmentation of mitochondria. On the contrary, mitochondria damaged by 3 Gy irradiation divided into punctate and clustered fragments, which were subsequently related to apoptotic cell death. The findings indicate that the degree of fragmentation determined mitochondrial damage responses and implies a stress response model to describe the distinct responses to mitochondrial damage caused by carbon ion irradiation with different doses in cancer cells.

A decrease of PARP-1 expression activates apoptotic signals through intrinsic and extrinsic pathways in un-irradiated cells. Carbon ion irradiation further enhances both the intrinsic and extrinsic pathways of apoptosis along with upregulation of p53 in HsiI cells resulting in overall higher apoptosis in HsiI than in H-vector. Based on the results described herein, mitochondria are the promising therapeutic targets for cancer treatments in relation to melatonin and carbon ion irradiation. Further preclinical and clinical investigations are indispensable in order to develop innovative anticancer agents.

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**Citation:** Liu M, Reiter RJ (2018) The Impact of Melatonin and Carbon Ion Irradiation on Mitochondria of Cancer Cells.SCIAEON J Radiol 1: 001-017.