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PK-PD based optimal dose and time for orally administered supra-pharmacological dose of melatonin to prevent radiation induced mortality in mice

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ABSTRACT

Aims: The study reports preclinical pharmacokinetics (PK) and correlation with pharmacological effect at supra-pharmacological dose of orally administered melatonin along with time and dose optimization, which have been lacking in earlier reports of radioprotection using melatonin.

Methods: PK of melatonin in C57BL/6 mice was evaluated after dose of 250 mg/kg using HPLC. Tissue distribution study was conducted in vital organs following oral administration. Plasma total antioxidant capacity (TAC) was determined by $ABTS^{\cdot+}$ radical assay and was correlated to plasma concentrations of melatonin. Using the outcomes of PK and Pharmacodynamics (PD), survival study was conducted for optimization of 'drug radiation gap period' (DRGP). Optimal oral dose for radioprotection was determined using survival as an end point.

Key findings: PK analysis of melatonin revealed T_{max} at 5 min with closely spaced another distinct concentration peak at 20 min. Plasma TAC of melatonin showed similar peaks at 5 min and 45 min, with the highest TAC at 45 min. Survival following a lethal (9 Gy) radiation dose was 20% and 40% after 5 and 45 min of melatonin administration, respectively. DRGP for melatonin was thus 45 min, while optimal oral dose ranged from 125 to 250 mg/kg. PK parameters at 250 mg/kg dose were qualitatively similar to low dose of melatonin, thus preventing chances of unexpected toxicity.

Significance: Survival enhancement at 45 min suggested as probable interval required as 'DRGP'. The optimum oral therapeutic window appears large with no substantial toxicity. The outcomes will be useful in development of radioprotectors as well as other therapeutic applications.

1. Background

Melatonin (*N*-acetyl-5-methoxytryptamine) is secreted from the pineal gland, and also synthesized in the gastrointestinal and other tissues in trace amount. Melatonin has multiple properties including free radical scavenger [1]. Several reports suggest that melatonin pretreatment protects biomolecules in cells from oxidative damages [2]. It enhances activity of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and inhibits action of pro-oxidant enzyme (nitric oxide synthase) [3,4]. Melatonin receptors are present in cells and have multiple roles, including protection of normal cells from oxidative damage for example by ionizing radiation [5,6].

Protection against radiation injuries during accidental exposure is an unmet medical need. Melatonin was first demonstrated for radioprotection by Blickenstaff et al. [7] and further by Vijayalaxmi et al. [8]. The beneficial effect of melatonin against radiation induced injury was confirmed in Swiss ND4 and CD2-F1 mice at 30 min intraperitoneal (i.p) preadministration [7,8]. Its radio-protective efficacy has been reported at varying doses in mice administered intraperitoneally 30 min prior to radiation [7–10].

A series of reports by Vijayalaxmi et al. [8,11,12], using cytogenetic assays in animal and human peripheral blood cells (in vitro) exposed to irradiation demonstrated protective effects of melatonin. Furthermore, in human study, after a 300 mg oral dose, the blood samples were

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collected and irradiated ex vivo and cytogenetic studies demonstrated beneficial effects of melatonin [11]. Investigation by Khan et al. has demonstrated that melatonin protects radiation-induced male testicular injury in C57BL/6 mice by enhancing regeneration of spermatogenic cells, and inhibiting lipid peroxidation, DNA strands break, and modulating ATM dependent p53-activation of pro-versus-anti-apoptotic proteins in whole body irradiated (WBI) mice [13]. Later, Khan et al. [9] further reported radio-protective role of melatonin administered intraperitoneally 30 min prior to whole-body gamma radiation exposure of 5 and 7.5 Gy in C57BL/6 mice. Melatonin (100 mg/kg, i.p) pre-treatment produced 100% survival in animals exposed to 7.5 Gy whole body radiation dose. Melatonin expands femoral hematopoietic progenitor stem cells and inhibits DNA strand breaks and apoptosis in spleenocytes of irradiated mice. Thus, leading to protection from radiation induced hematopoietic, immunological, and gastrointestinal injury, thereby overcoming lethality in mice. These results from our laboratory and other published reports strongly suggest the potential of melatonin in developing as a radio-protector product for planned and accidental radiation exposure [2,9–11].

In addition, during the last 3 decades, numerous clinical trials have examined the therapeutic usefulness of melatonin in different indications using a variety of oral dosage forms of melatonin. For example melatonin 5–20 mg has been used clinically in insomnia [14]. The clinical PK of exogenously administered melatonin is variable, contributing to inconclusive results in clinical trials [15–18]. Melatonin has been formulated in various pharmaceutical dosage forms for different experiments and has contributed to the variability in PK. Pharmacokinetic studies have reported melatonin doses ranging from 0.3 to 100 mg/subject [17]. Variable oral bioavailability in humans (9%–33%) with inconsistent T_{max} ranging between 15 min (at 2 mg dose) to 210 min (at 10 mg dose) can be observed. Also, preclinical pharmacokinetic studies have reported melatonin doses ranging from 1 to 10 mg/kg in mice [19,20], that provided varying physiological/pharmacological concentrations of melatonin. These variabilities add to the challenges in designing of experiments involving melatonin in radioprotection [17–21]. Thus, a detailed investigation of the PK of melatonin in preclinical models is necessary before translation to clinical use.

It is hypothesized that high dose melatonin may be developed for clinical use in radiation injury/radiotherapy. Since the pioneering reports [7,8], a number of reports have recommended melatonin for radioprotector development [9,13,22]. The PK of such supra-pharmacological doses of melatonin (> 100 mg/kg in mice) has not been reported. As PK of melatonin is variable, dose and species dependant, it makes it necessary to understand PK of high dose melatonin in rodent models. Therefore, the current study was planned to elucidate the PK and tissue distribution of melatonin administered orally in mice at doses of 250 mg/kg.

In addition, the time interval necessary for effectiveness of melatonin prior to radiation exposure is still uncertain because of poor understanding of pharmacokinetics–pharmacodynamics (PK-PD) correlation. Radioprotection by an investigational drug molecule is an outcome of multiple properties of the molecule, because radiation targets all tissues and organs. Free radical scavenging appears to be first line of action by the molecule followed by various cellular response parameters in the presence of melatonin. Therefore, assessment of the time interval between melatonin administration and radiation exposure is important. Thus, the present study focused on the time of drug administration prior to radiation exposure, “drug radiation gap period” (DRGP). The plasma concentration and anti-oxidant capacity peaks were also matched to optimize the time of administration of melatonin before radiation injury. The protective action of oral melatonin at optimized time of administration was evaluated in 7.5 Gy and 9.0 Gy irradiated C57BL/6 female mice. Furthermore the dose of oral administration was optimized using survival against lethal radiation exposure as endpoint.

2. Material and methods

2.1. Chemicals

Melatonin, Hesperetin, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulphate, EDTA were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Acetonitrile, methanol and glacial acetic acid (HPLC grade) were obtained from Spectrochem Pvt. Ltd., India. Polyethylene glycol (PEG)-400 was procured from SD Fine Chem Ltd., India.

2.2. Animals

Female C57BL/6 mice (age 8–10 weeks) were obtained from experimental animal facility, Institute of Nuclear Medicine & Allied Sciences (INMAS), Delhi, India. Mice were housed in cages under optimum conditions of temperature (25 °C ± 2 °C), humidity (50–60%) and light (14 h of light and 10 h of dark), and provided with standard food and water ad libitum. All necessary methods and protocol were approved by the institutional animal ethics committee. (INM/IAEC/2012/06).

2.3. Pharmacokinetic evaluation

2.3.1. Drug administration

Melatonin PK was evaluated after oral administration in two drug formulations and was compared for bioavailability against intravenous administration. Thus, melatonin was administered either as (i) an oral aqueous suspension (Melatonin aqueous suspension at a dose of 250 mg/kg was prepared as a suspension in water 400 μL/mice, freshly prepared and shaken well prior to oral administration via gavage tube) or (ii) an oral solution in PEG-400 (Melatonin in PEG-400 at a dose of 250 mg/kg, first dissolved in PEG-400 20% w/v and further diluted with water 400 μL/mice, freshly prepared and administered orally via gavage tube) or (iii) intravenous solution (Melatonin at a dose of 62.5 mg/kg, dissolved in water 125 μL/mice to get a clear solution and administered intravenously through tail vein).

2.3.2. Blood sample collection

Ninety animals were weighed, matched and divided into 6 different groups of five animals for each treatment (n = 30), i.e., three different pharmacokinetic evaluation were conducted. Blood samples (150 μl) were collected from retro-orbital plexus at 0, 5, 10, 15, 20, 30, 45, 60, 90 min and 2, 3, 4, 6, 8, 12 h after melatonin administration. Each mouse was bled thrice to collect samples for all time points. Blood was collected in K-EDTA microtainer, preserved on wet ice till plasma was separated after centrifugation at 2000*g*, 4 °C, for 15 min. The supernatant plasma was stored in 50 μl aliquots for HPLC analysis and 20 μL aliquots for ABTS assay at −80 °C until analysis.

2.3.3. Tissue distribution

Tissue distribution of melatonin was assessed after melatonin formulation containing PEG-400 administered orally. The animals of the pharmacokinetic study were terminally sacrificed at specified time points and the blood samples were obtained from cardiac puncture. The time points for tissue distribution were (0.5, 1, 2, 4, 8, 12 h) of five mice each (n = 5) to collect brain, heart, lungs, liver, stomach, intestine, spleen and kidney. All the organs were trimmed to remove attached subcutaneous fats and then washed with pre-chilled phosphate buffered saline; the organs were then preserved at −80 °C until analysis.

2.3.4. Plasma sample preparation for HPLC analysis

Stored samples were allowed to thaw and each 50 μL aliquot was treated with 200 μl of chilled acetonitrile, containing hesperetin (2.5 μg/mL, internal standard (IS)). Precipitated samples were centrifuged at 10,000*g*, 4 °C for 15 min (Hermle, Z-326K, Germany) and

20 μL of supernatant was injected for analysis using HPLC-UV detection (Ultimate 3000, Thermofischer Scientific).

2.3.5. Tissue sample preparation for HPLC analysis

Tissue homogenate (50% w/v) was prepared in 50 mM sodium acetate buffer (pH 4.3) containing 0.1 mM disodium EDTA using tissue homogenizer (Omni TH, Omni International, USA) and 200 μL of homogenate was treated with 800 μL of Hesperetin in acetonitrile, 2.5 μg/ml. Precipitated samples were centrifuged at 10,000*g*, 4 °C for 15 min and 20 μL was injected for analysis using HPLC-UV detection.

2.3.6. Chromatographic conditions

The HPLC separation and quantification was achieved on a 240 × 4.6 mm (I.D), 120 Å, 5 μ Acclaim® reverse phase C-18 column. Mobile phase for plasma sample analysis consisted of 0.1% acetic acid and acetonitrile (60:40, V/V), while for tissue sample analysis it was 50 mM sodium acetate buffer (pH 4.3) with 0.1 mM disodium EDTA and acetonitrile. The flow rate was 1.0 mL/min, column temperature 35 °C, with Chromatograms recorded at 278 nm. For linearity curve, melatonin was dissolved in drug free plasma in the range of 195 ng/ml to 200 μg/ml. The peaks of melatonin and hesperetin were detected and peak area ratios were subjected to regression analysis for linearity curve.

2.4. Pharmacokinetic analysis

PK parameters were calculated using PK solver 2.0, with linear trapezoidal method for non-compartmental analysis of plasma data after extravascular Input [23]. C_{max}, T_{max}, Cl, K_{el}, AUC, and bioavailability, after oral administration of PEG formulation and suspension, and IV solution were analyzed. Absolute bioavailability was measured by comparing the respective AUCs after intravenous and oral administration according to the following equation

$$F = \frac{AUC_{PO}/Dose_{PO}}{AUC_{IV}/Dose_{IV}}$$

2.5. Total antioxidant capacity

The pre-formed radical monocation of ($ABTS^{\cdot +}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of antioxidants. The total antioxidant capacity was determined by decolourization of $ABTS^{\cdot +}$ through measuring % decrease in absorbance [24], for each plasma sample obtained after administration of melatonin in PEG-400. Absorbance was recorded at 732 nm for 60 min using kinetic scan in microplate reader (VarioskanFlash, Thermofisher, Germany).

2.6. DRGP optimization

Animal Survival was recorded as an indication of radioprotection efficacy and C57BL/6 mice are recommended animal model for evaluation [25,26]. Mice were placed in a plexi-glass box to accommodate 5 mice during each exposure at 1 Gy/min dose rate using a calibrated ^{60}Co teletherapy unit (Bhabatron II, Panacea, India). WBI delivered a total dose of 9 Gy (lethal, $LD_{100/30,}$ Radiation dose to cause 100% lethality in observation period of 30 days) and 7.5 Gy (sub-lethal, $LD_{50/30}$) in different groups of mice as mentioned below. The animals were monitored for 30 days for any mortality.

Mice were divided into 7 groups mentioned below.

Group A: Melatonin in PEG-400 (20%), 250 mg/kg at 45 min prior to 9 Gy radiation
Group B: Melatonin in PEG400 (20%), 250 mg/kg at 5 min prior to 9 Gy radiation
Group C: PEG-400 (20%) in water at 45 min prior to 9 Gy radiation
Group D: PEG-400 (20%) in water at 5 min prior to 9 Gy radiation
Group E: 9 Gy radiation only
Group F: Melatonin in PEG-400 (20%), 250 mg/kg at 45 min prior to 7.5 Gy radiation
Group G: 7.5 Gy radiation only

2.7. Melatonin oral dose optimization

Melatonin was administered in different doses (500, 250, 125, 62.5, 31.25 mg/kg) taking 45 min DRGP, and exposed to lethal radiation ($LD_{100/30}$) at 1 Gy/min dose rate using a calibrated ^{60}Co teletherapy unit (Bhabatron II, Panacea, India). This was followed by a 30 day monitoring for survival.

2.8. Statistical analysis

One way ANOVA followed by Dunnet's multiple comparison and two way ANOVA followed by Benferroni post-test were used for antioxidant capacity and PK analysis at different time points, respectively using graph pad prism. Data are expressed as mean ± standard deviation (SD) and $P < 0.05$ was considered as significant. Survival analysis was performed by Kaplan Meier plot with log rank (mental cox) test using graph pad prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

3. Results

3.1. Calibration curve (LOQ and linearity)

Melatonin with hesperetin in plasma exhibited chromatographic separation during a run-time of 13 min. The retention times of melatonin and the IS were 4.8 and 10.1 min, respectively. Fig. 1A presents the chromatograms of plasma spiked with melatonin and internal standard (IS). The limit of quantification (LOQ) of melatonin was 195 ng/mL with the lowest detection limit (LOD) as 90 ng/mL. A linear standard curve was obtained by analysing a series of samples with concentrations in the range 0.19–200 μg/mL (Fig. 1B). The linearity equation was y = 0.034x + 0.045. Reproducibility of melatonin estimation was monitored and was found within predefined acceptable limits.

3.2. Pharmacokinetic study

After oral melatonin aqueous suspension (250 mg/kg), the mean C_{max} was 59.82 ± 3.3 μg/mL at 10 ± 4.1 min (T_{max}) with AUC_{0-t} of 1766.05 ± 325.66 min·μg/mL. Elimination $T_{1/2}$ and clearance were 40.91 ± 2.8 min and 3.4 ± 0.6 mL/min respectively (Fig. 2A). After oral melatonin in PEG-400 at an oral dose of 250 mg/kg in C57BL/6 mice, C_{max} of 199.09 ± 37.51 μg/mL was obtained at 7 ± 4.47 min (T_{max}). The AUC_{0-t} and $T_{1/2}$ were 6006.33 ± 644.43 min·μg/mL and 47.95 ± 36.62 mL/min respectively and rate of clearance was 0.96 ± 0.09 ml/min (Fig. 2B).

Following i.v administration of melatonin 62.5 mg/kg, C_{max} attained was 87.23 ± 9.03 μg/mL at 5 min (T_{max}) with AUC_{0-t} of 1503 ± 298.8 min·μg/mL, $T_{1/2}$ and clearance were 46.86 ± 15.19 min and 1.06 ± 0.2 mL/min respectively (Fig. 2C). Table 1 shows all the PK parameters obtained after melatonin administration by various routes and formulation. The oral bioavailability of melatonin in aqueous suspension and PEG formulation were 29% and 98.5% respectively.

3.3. Tissue distribution

Melatonin was widely distributed in all body tissues; the tissue concentrations detected were much higher than the physiological concentrations of melatonin. Highest concentrations of melatonin were

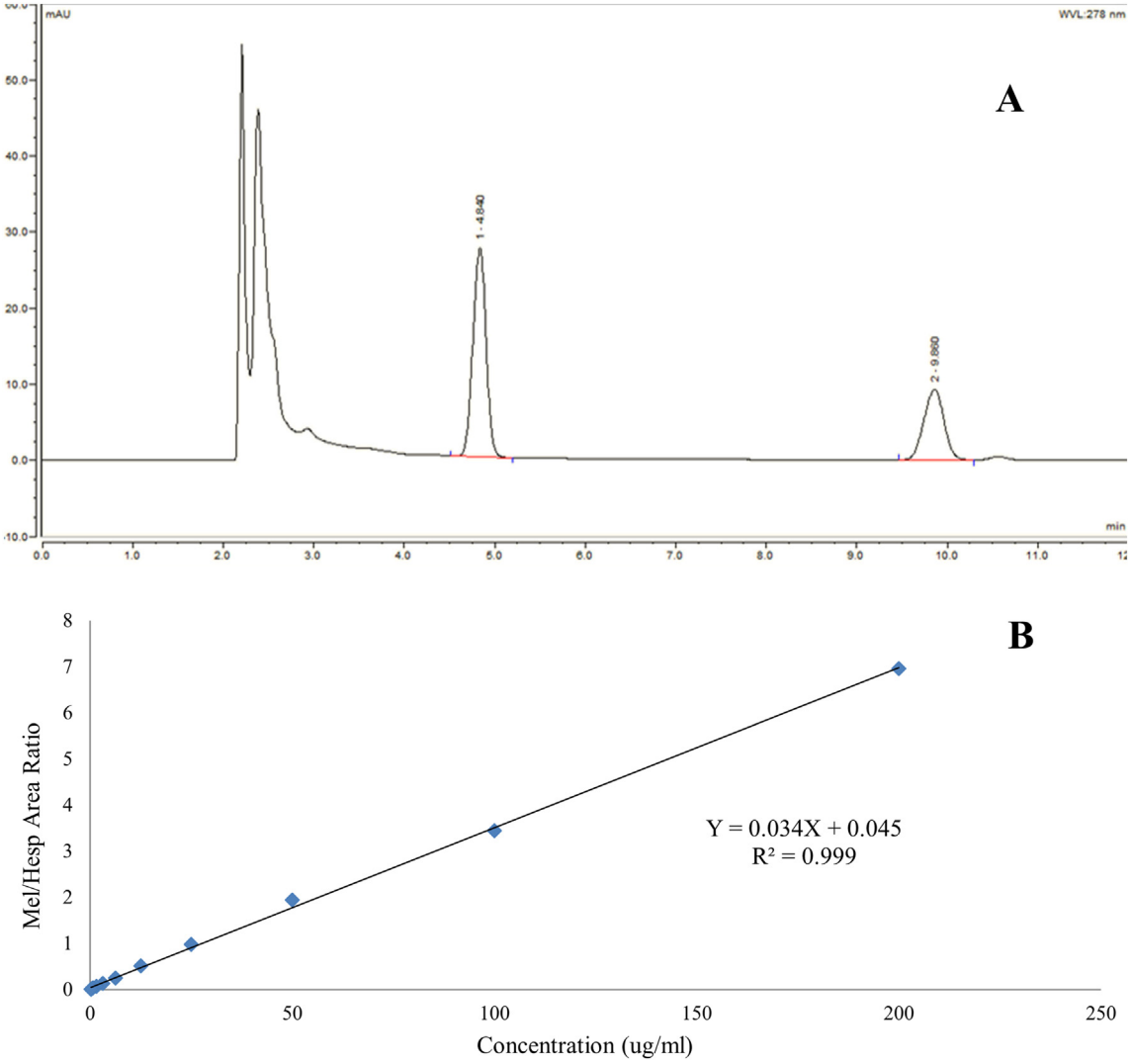

Fig. 1. (A) Chromatogram of plasma spiked with melatonin and internal standard (IS; Hesperetin), (B) Linearity curve of melatonin Area ratio v/s concentration (μg/ml); Area Ratio = area Melatonin Peak/area Hesperetin Peak.

observed in stomach and lowest in brain. The highest concentrations obtained were 3.13 μg/mg (stomach), 1.04 μg/mg (intestine), 0.26 μg/mg (liver) at 30 min after administration. In other organs, this value was 0.20 μg/mg (lungs), 0.17 μg/mg (kidney), 0.13 μg/mg (heart), 0.11 μg/mg (spleen), and 0.08 μg/mg (brain) after 60 min of melatonin oral administration (Fig. 3A and B). Total drug exposure to a particular tissue over a period of time was determined using area under curve (AUC), the data is shown in Table 2.

3.4. Total antioxidant capacity and PK-PD correlation

The total antioxidant capacity in plasma of mice after oral administration of single dose of 250 mg/kg melatonin in PEG was maximum at 5 min and 45 min post-treatment. The rise in antioxidant capacity was approximately 45% as compared to basal antioxidant capacity (Fig. 4). The percentage change in total antioxidant capacity was plotted against plasma concentration measured at the same time and a best-fit line with equation $y = 0.187x + 11.73$ with an R^2 of 0.517 (Fig. 5).

3.5. DRGP optimization

Fig. 6 depicts that Group A (Mel 45 min) had significantly higher survival than group E (radiation alone) ($P < 0.05$), i.e., 40% and 0% respectively, while melatonin administration 5 min (Group B) prior to radiation had lower effect (20%) on survival enhancement. At sublethal radiation dose the drug treatment showed 100% survival in comparison to 50% survival of radiation (7.5 Gy) group (Fig. 7).

3.6. Melatonin oral dose optimization

Melatonin showed highest survival with 500 mg/kg, i.e., 55%, followed by 125 and 250 mg/kg to be 40%. Other lower doses had lower survival as compared to 125 mg/kg. (Fig. 8) Thus 125 mg/kg appears to be optimal dose for radioprotection in mice model.

4. Discussion

PK of molecules is necessary for radioprotector development and very few studies have reached this level in radiation counter measure development. On the contrary, the number of discovery molecules is increasing but PK studies are lacking in literature, two recent reports on bisbenzoimadazole [27] and δ tocotrienol [28] are worth mentioning. The former being DNA ligand and PK established through scintigraphy technique. PK of tocotrienol is studied using HPLC. PK of melatonin administered in rats, dogs and monkeys at 10 mg/kg have been

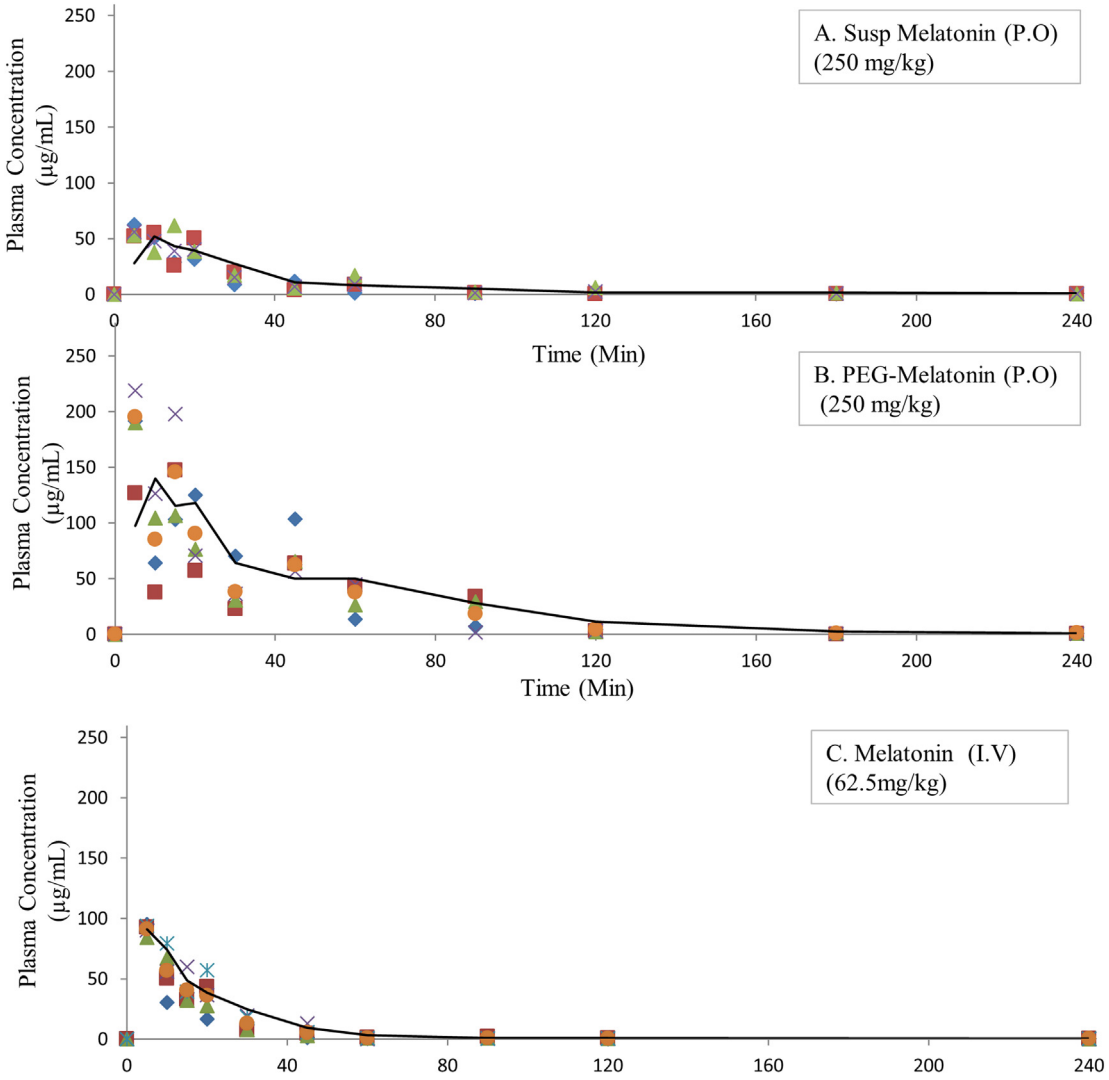

Fig. 2. Shows the pharmacokinetics of melatonin (plasma concentration (µg/ml) v/s time) for 240 min in C57BL/6 mice (n = 5 for each concentration point), (A) after administration of melatonin 250 mg/kg suspension (P.O), (B) melatonin 250 mg/kg in PEG (P.O), (C) melatonin 62.5 mg/kg (I.V.)

reported. Clinical studies have reported human PK at doses up to 100 mg orally, and the different PK parameters were found to be affected by factors such as age, gender, genotype [15–18,29]. In animals, melatonin PK has been shown to vary with strain, species, first pass metabolism after administration at doses ranging from 2 µg/rat to 10 mg/kg body weight [19,20,30,31]. In preclinical model, Li et al., reported T_{max} of 15 min with C_{max} 168.86 ng/ml when dosed with 2 mg/kg melatonin in Sprague Dawley rats [20]. In another study, Yeleswaram et al. reported PK of melatonin [19] in rat, dogs and monkey at 3–10 mg/kg with highly variable oral bioavailability ranging within 16–100%. In rats, oral administration of 10 mg/kg melatonin led to a C_{max} of about 5 µg/ml with a half-life of 20 min and bioavailability of 53.5%. In another study, 2 µg melatonin was infused in rats and was found to provide a C_{max} of 800 pg/ml, with a half life of approx 20 min [30] while administration of 100 µg melatonin intraperitoneally in rats, reported approx 240 ng/mL C_{max} with a half-life of about 22.5 min [31].

In the present study, the two types of oral doses administered to mice were at 250 mg/kg and following adjustment of dose for oral and intravenous administration, the PK parameters of elimination half-life

Table 1
Summary of Pharmacokinetic parameters of melatonin form different formulation and routes in c57Bl/6 mice (Data represented as Mean ± S.D, n = 5).

Parameter	IV	Oral suspension	Oral PEG
Dose (mg/kg)	62.50	250.00	250.00
C_{max} (ug/ml)	87.92 ± 9.03	59.82 ± 4.00	199.10 ± 37.51
T_{max} (min)	5.00 ± 0.00	10.00 ± 4.08	7.00 ± 4.47
AUC 0 − t (min·ug/ml)	1503.08 ± 298.8	1766.05 ± 325.6	6006.33 ± 644.4
K_{el} (1/min)	0.019 ± 0.008	0.017 ± 0.001	0.014 ± 0.007
$T_{1/2}$ (min)	46.8 ± 15.2	40.91 ± 2.88	47.95 ± 36.60
AUC 0 − ∞ (min·ug/ml)	1528.28 ± 301.83	1773.89 ± 330.79	6026.69 ± 641.77
Clearence (ml/min)	1.06 ± 0.20	3.4 ± 0.6	0.96 ± 0.09
Bioavailability	100.00%	29.02%	98.59%

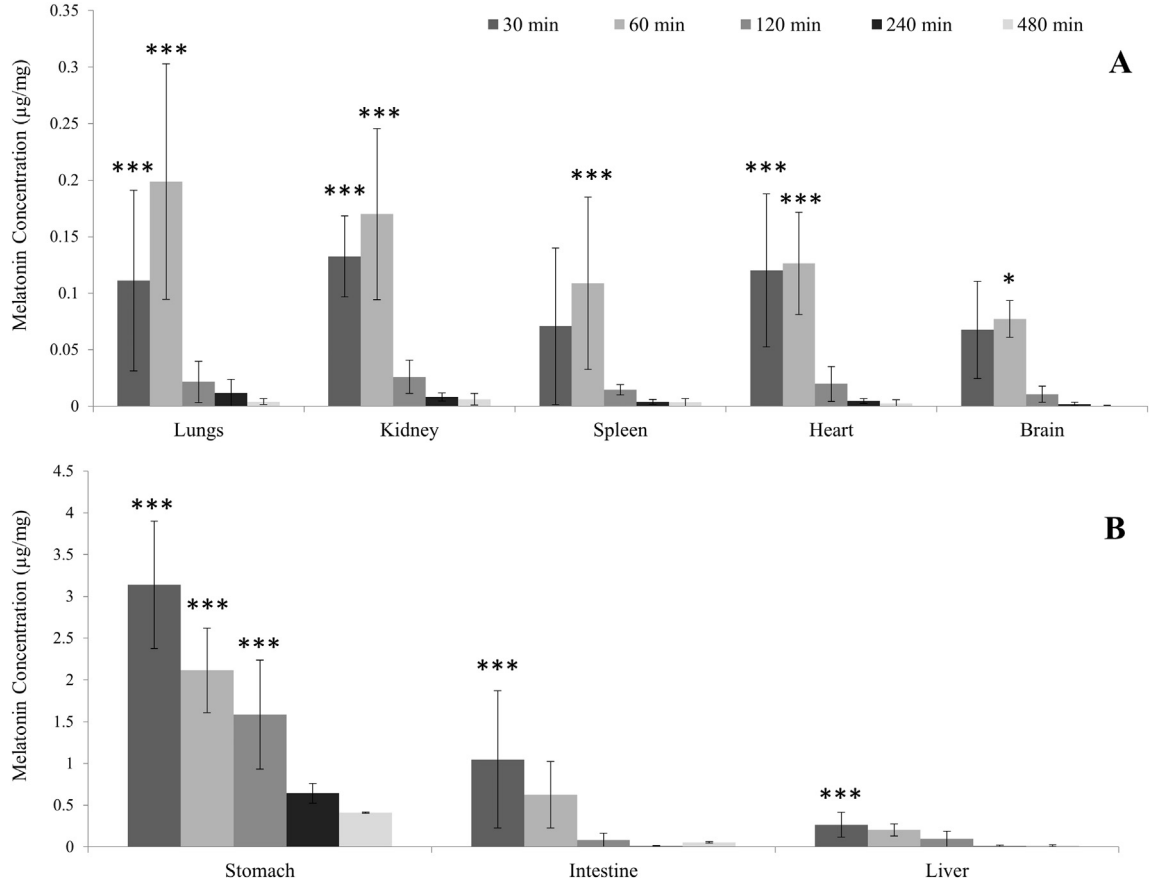

Fig. 3. Biodistribution of melatonin in PEG (20%) in C57BL/6 mice (n = 5 for each concentration point) (A) biodistribution in lungs, kidney, spleen, heart, brain, (B) biodistribution in intestine, stomach and liver. (*P < 0.05, ***P < 0.001, compared to respective tissue concentration at 480 min).

Table 2
Biodistribution represented by Area Under Curve (AUC) of melatonin PEG in C57Bl/6 mice tissues (Data represented as Mean ± S.D, n = 5).

Organ	AUC 0 − t (min·ug/ml) Mean ± SD
Intestine	51.98 ± 29.14
Stomach	323.43 ± 60.60
Liver	22.52 ± 11.81
Kidney	12.47 ± 3.51
Spleen	7.50 ± 4.24
Lungs	13.25 ± 8.65
Heart	9.54 ± 3.50
Brain	8.64 ± 1.72

and clearance were similar to those reported after administration of lower doses. This implies that very high doses of melatonin are eliminated from the body at the same rates as observed at lower doses and unlikely to produce unexpected toxicity.

In this study, the oral PEG formulation prepared to provide a dose of 250 mg/kg, demonstrated enhanced bioavailability (98%) and caused a threefold rise in C_{max} and AUC as compared to oral suspension (29% bioavailability). Melatonin is slightly soluble in water, 250 mg/kg dose of melatonin formed a suspension when attempted to dissolve in water and hence PEG-400 was used as co-solvent for solubilising melatonin and to enhance the bioavailability. The PEG formulation was prepared as a solution of 15 mg/ml melatonin in 20% PEG as vehicle and the resultant dose of PEG received by each mouse in from the formulation was 3.2 g/kg. PEG is commonly used as safe vehicle for compounds in drug discovery research, its limit for use in oral solution is 40% (w/v) in USFDA inactive ingredient database [32].

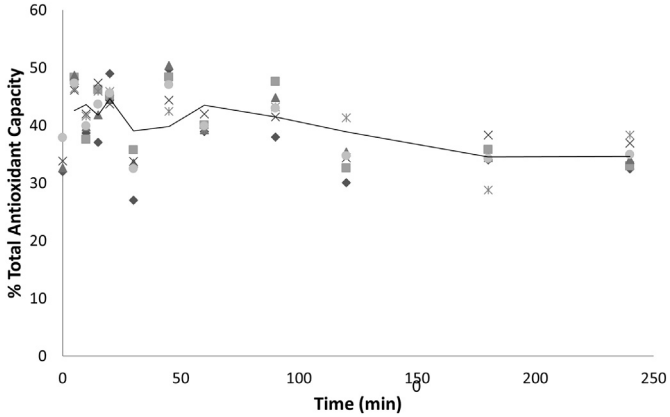

Fig. 4. Total antioxidant capacity in Plasma after melatonin administration (250 mg/kg) v/s time (min) in C57BL/6 mice (n = 5). Plasma TAC was estimated using ABTS assay.

Melatonin being lipophilic in nature crosses all physiological barriers, tissues of body, within short period of time [33]. Report of melatonin tissue distribution available in the literature has shown distribution of melatonin after intravenous administration using radioactive melatonin. In the present study, after oral administration, melatonin was found to be distributed among all vital organs namely gastrointestinal tract, liver, lungs, kidney, heart, spleen and brain and maximum tissue concentrations were observed between 30 and 60 min after melatonin administration. The concentration of melatonin in the gastrointestinal tract and liver were 5 to 10-fold greater than that observed in lungs and other vital organs while being devoid of the luminal

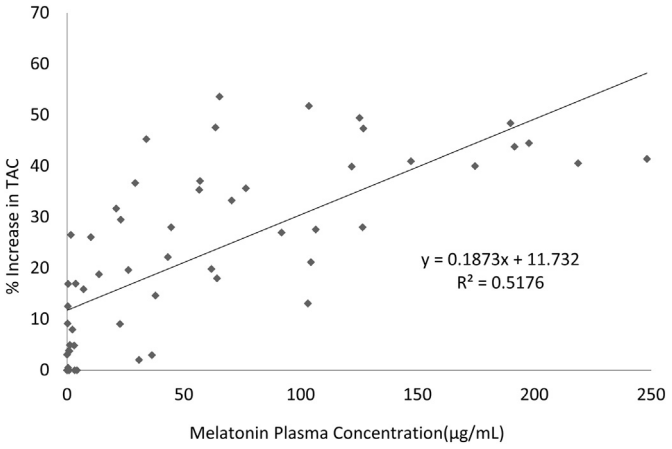

Fig. 5. PK-PD correlation, the percentage change in total antioxidant capacity is plotted against concomitant plasma concentration with a best-fit line. (Percentage change in TAC was taken as pharmacodynamic measure).

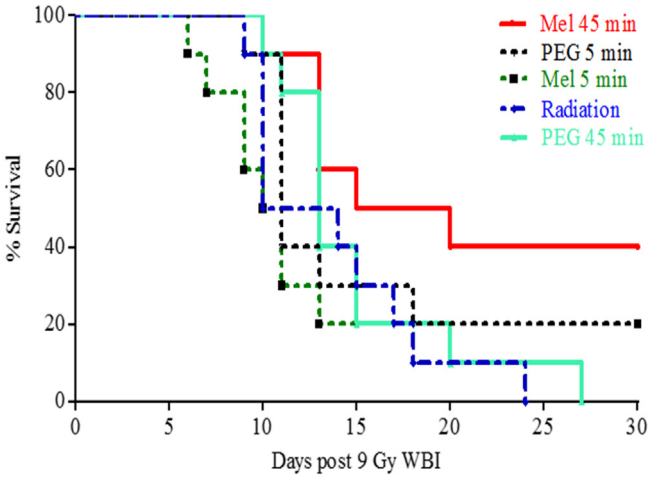

Fig. 6. Mice (n = 10 in each group) underwent $LD_{100/30}$ C^{60} γ-radiation exposure (9 Gy), with melatonin (250 mg/kg) preadministered at 5 min and 45 min, preadministration of vehicle at 5 min and 45 min, and radiation alone. Survival was noted during the following 30 days.

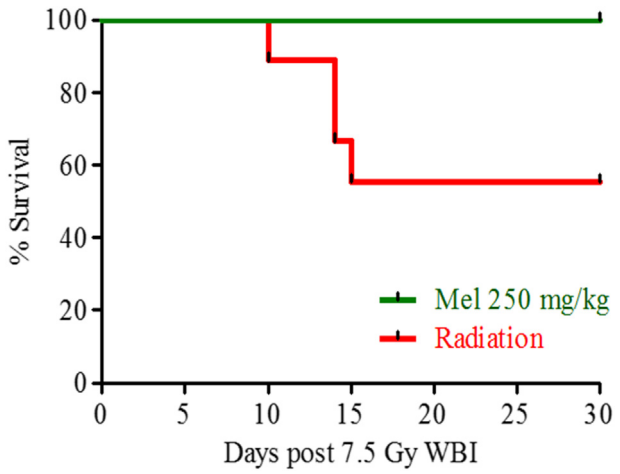

Fig. 7. Mice (n = 10 in each group) exposed to $LD_{50/30}$ C^{60} γ-radiation (7.5 Gy), with or without preadministration of melatonin (250 mg/kg), Survival was noted during the following 30 days. Kaplan-Meier plot shows survival at 50% in radiation alone and 100% after melatonin pretreatment.

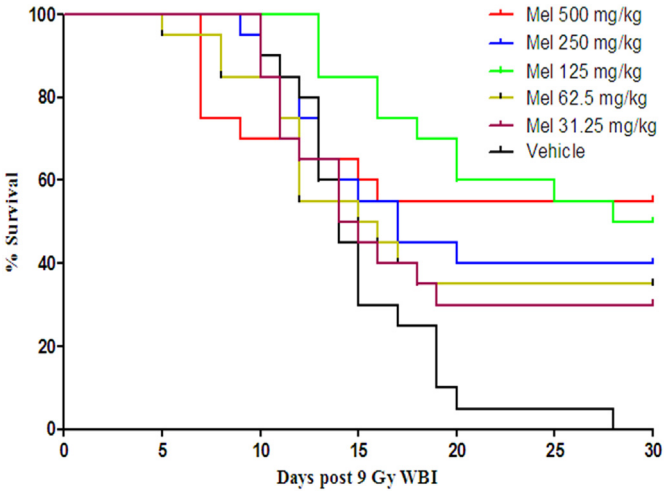

Fig. 8. Mice (n = 20 in each group) exposed to $LD_{50/30}$ C^{60} γ-radiation (9 Gy), with melatonin preadministration at different dose, Survival was noted during the following 30 days. Kaplan-Meier plot shows survival after melatonin pretreatment at different doses (500, 250, 125, 62.5, 31.25 mg/kg body wt.).

content of the gut. This high concentration in gut and the liver may be indicative of a high first pass metabolism of melatonin.

Melatonin concentrations have shown two peaks in the first 1–2 h after oral administration. The reason for double peak phenomena is not known, rapid redistribution of melatonin into organs and body tissues [21] have been proposed to contribute to such an effect. Alternatively, the variability in absorption from different parts of the gut may also contribute to this effect. Enterohepatic recycling is commonly considered as an underlying mechanism [34] for multiple peaks, the peaks of melatonin were observed in the first 60 min after drug administration and could be related to this phenomenon, supported by high concentration of melatonin in GI and liver. The therapeutic implication of the double-peak phenomenon observed in the present study needs further evaluation.

Earlier studies have reported that systemic melatonin appears to ameliorate radiation-induced injury in various organs including the spleen, liver, lung, gut, kidney, lens, spinal cord and brain [35]. The present data clearly demonstrates that orally administered melatonin reaches wide variety of tissues and organs, which could contribute to enhancing plasma total antioxidant capacity thereby providing protection against WBI.

Melatonin concentrations were measured in plasma and tissues after drug administration and were correlated to PD effects of total antioxidant capacity, though survival after radiation injury was also assessed as a qualitative measure. It exhibits antioxidant effects and performs receptor independent metabolic functions, i.e., it is a multifaceted scavenger of free radicals [36,37]. Radioprotection as being considered to depend on the melatonin concentration in body i.e., due to chemical interaction of melatonin to free radicals, quantification of its concentration in vital tissues becomes necessary. However, the plasma concentration-antioxidant capacity due to melatonin demonstrated a moderately increasing dose-response plot, characteristic of a non-receptor mediated action of the drug, with a ceiling of 50% increase in TAC with plasma concentration above 200 μg/ml.

When the concentration of melatonin and the plasma antioxidant capacity plotted serially, a PK-PD curve tending towards a counter-clock hysteresis loop is observed. This indicates that there is a delay in biological activity after achieving adequate concentration of the drug which could be due to the indirect mechanism of action of melatonin. The PD measure, i.e. TAC, here was measured in the plasma and found to be maximum at 45 min after drug administration. However, a plasma concentration maximum is achieved at 7 min with tissue distribution of melatonin occurring rapidly with peak organ concentrations between

30 and 60 min. Alternatively, the mismatch in the melatonin concentration effect could be contributed by the active metabolites of melatonin (*N*-Acetyl-*N*-formyl-5-methoxykynurenamine, N1-acetyl-5-methoxykynuramine) [38] or the activation of an endogenous antioxidant system [2].

Plasma concentrations of melatonin peaked earlier than antioxidant capacity peak, while enhanced survival after lethal radiation exposure was observed in accordance to antioxidant peak. Enhancement of survival against lethal radiation dose in mice is an important consideration in preclinical evaluation of radio-protector research [26]. Melatonin has been investigated earlier for its radioprotective efficacy through intraperitoneal route by Vijaylakshmi et al. [2] and others [9,39], and significant enhancement in animal survival was reported, though, the dose and time of administration prior to radiation exposure remained questionable. Radioprotector is envisaged for administration during planned exposure to radiation emergency responders and the intended route of administration is oral [40]. The present study confirmed the earlier results through oral administration and identifies 45 min as the probable interval required as DRGP.

5. Conclusion

Thus, melatonin (250 mg/kg), a drug of interest for radiation protection, which could be used in emergency situations and during radiotherapy, demonstrated 40% survival efficacy when administered 45 min before lethal WBI and 100% efficacy with sub-lethal WBI in mice. The optimal dose for orally administered melatonin in PEG-400 ranges from 125 to 250 mg/kg. The PK of this high dose melatonin in plasma and in organs was qualitatively similar to PK of lower doses of melatonin. Melatonin administration also improved the total antioxidant capacity and this contributes to benefits in WBI; the correlation of this effect to the plasma concentration was moderately increasing with melatonin plasma concentration.

Abbreviations

ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AUC	Area under curve
DRGP	Drug Radiation Gap Period
Gy	Gray (Radiation unit)
i.p	Intra-peritoneal
IS	Internal Standard
PBS	Phosphate-Buffered Saline
PEG	Polyethylene Glycol
PK-PD	Pharmacokinetics-Pharmacodynamics
TAC	Total Antioxidant Capacity
WBI	Whole Body Irradiation

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