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Melanin nanoparticles: Antioxidant activities and effects on γ-ray-induced DNA damage in the mouse

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ABSTRACT

The radioprotective and antioxidant activities of melanin nanoparticles (MNP) were investigated in Chinese hamster ovary (CHO) cells in vitro and BALB/C mice in vivo. The endpoints measured were cell viability, superoxide dismutase (SOD) enzyme activity, malondialdehyde (MDA) levels, DNA damage (comet assay), and histopathological examination of tissues. Irradiated groups showed decreased SOD activity and increased MDA levels. Irradiation caused a 3–10-fold increase in comet parameters such as % tail DNA. Treatment with MNP protected cells from DNA damage and death, restored SOD activity, and decreased MDA production. Synthetic MNPs have both antioxidant and radioprotective activities.

1. Introduction

In biological systems, ionizing radiation creates free radicals and reactive oxygen species (ROS) [1] which can attack DNA, protein, and membranes lipids [2]. The effects of ROS are mitigated by antioxidant defenses, including glutathione, ascorbic acid, uric acid and enzymes such as catalase, superoxide dismutase (SOD), and glutathione peroxidase [3–5]. Excess ROS or depletion of antioxidant defenses can lead to oxidative stress.

Oxidative stress is implicated in pathological conditions, including hepatotoxicity [6], nephrotoxicity [7] and cardiovascular disease. ROS and reactive nitrogen species (RNS) contribute to the development of many chronic diseases [8] and Phiwayinkosi et al., 2017. ROS attacks DNA, leading to single- and double- strand breaks, base losses, and base modifications. Despite repair mechanisms, ROS can cause permanent changes to DNA, with detrimental effects on cells [9–11].

Protection of biological systems from the consequences of ionizing radiation exposure (whether accidental or unavoidable) is important [12]. Novel approaches for radioprotection are of interest for defense, the nuclear industry, space travels, and the protection of normal tissues during cancer radiotherapy. Many natural and synthetic compounds have been investigated for their ability to protect against irradiation damage [13]. However, the toxicity of some synthetic agents has prompted further examination of natural radioprotectors.

Melanins are high molecular weight pigments found in all biological kingdoms, providing protection from visible and ultraviolet light and oxidative stress [14–17]. Melanins are polymers with quinone components that contribute to their redox behavior. The task of a radioprotector may be to counteract the oxidizing effects of ionizing radiation without bleaching Charles et al. [18].

Melanin can protect pigment cells against oxidative stress by scavenging ROS [19–23]. Kunwar et al. [24] confirmed the radioprotective activity of fungal melanin against whole-body irradiation (WBI) and concluded that the mechanisms of radioprotection involve modulation of pro-survival signaling, prevention of oxidative stress, and immunomodulation. Melanin nanoparticles (MNPs) produced by oxidation of dopamine under basic conditions were nontoxic and gave excellent radioprotection against WBI [25–27].

Here, we have studied the radioprotective effects of MNP against γ-irradiation induced oxidative damage in vitro and in the liver, heart, and kidney of the mouse.

2. Materials and methods

2.1. Materials

Methoxypolyethylene glycol thiol (mPEG-SH, MW5000),dopamine (3.4- dihydroxyphenethylamine HCl), 1 N NaOH solution, MDA Assay kit and SOD determination kit were obtained from Sigma-Aldrich (Schnelldorf, Germany) and phosphate buffer saline (PBS) from MP Biomedicals (Santa Ana, CA USA).

2.2. Preparation of melanin nanoparticles

MNP were prepared by the methods of [28,29]. Briefly, dopamine HCl (90 mg) was dissolved in deionized water (45 mL) and 1 N NaOH

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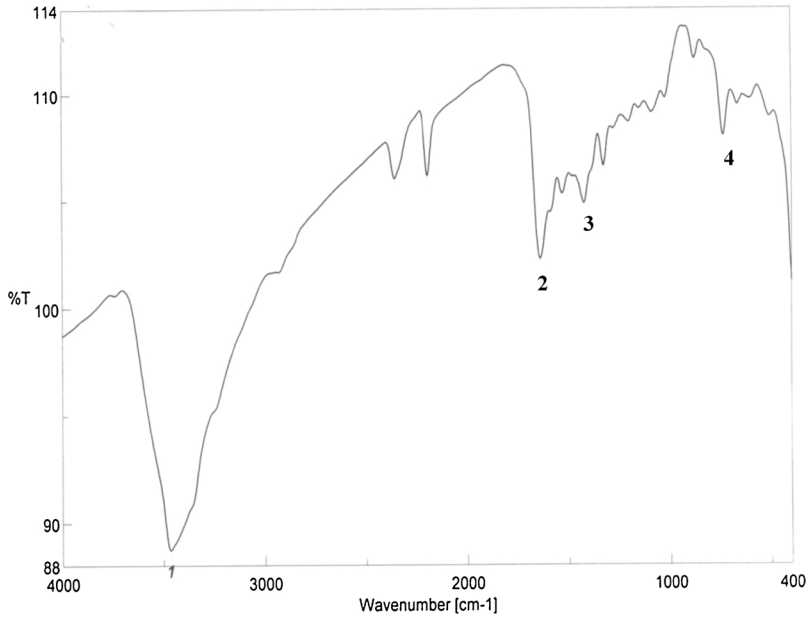

Fig. 1. FT-IR spectra of melanin nanoparticles (MNPs).

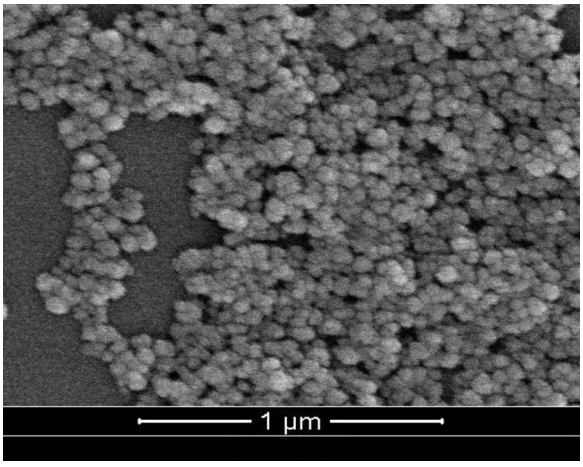

Fig. 2. Scanning electron microscope (SEM) image of melanin nanoparticles (MNPs).

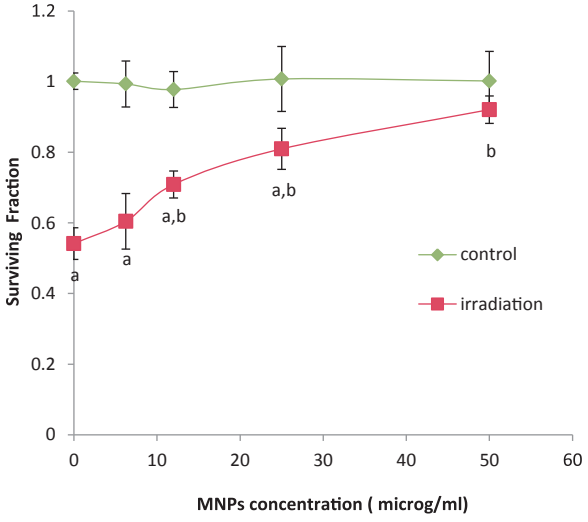

Fig. 3. Effect of varying concentration of MNPs against gamma- irradiated cells (6 Gy) induced cytotoxicity in CHO cells. The data point are represented as mean ± SD (n = 3). (a-p ≤ 0.05) when compared to control group (zero conc. of MNPs),b- p ≤ 0.05 when compared to irradiated group (zero conc. of MNPs).

(380 µL) was added with stirring, for neutralization. The pale yellow solution was stirred on a hot plate stirrer (MS-300HS, Misung Scientific, Gyeonggi-do, Korea) at 50° C for 5 h for polymerization; the color gradually deepened. The product was purified by centrifugation three times at 6600 x g for 30 min. MNP were recovered by centrifugation at 620 x g for 10 min.

PEG-modified MNP were prepared as follows. A dispersion of MNPs (1 mg/ml) was adjusted to pH 10.5 with 1 N NaOH and mixed with methoxypolyethylene glycol thiol (mPEG-SH, MW5000) at molar ratio MNP: mPEG-SH = 1:3 according to the methods of [29].

The resulting suspension was centrifuged three times at 6600*g* for 30 min. Modified MNP dispersed in PBS buffer were obtained after centrifugation at 620*g* for 10 min. The modified MNP were prepared fresh before injection into mice (dose, 50 mg/kg body weight).

2.3. FT-IR spectroscopy

A Fourier- transform IR spectrometer (Basic Vector, 22FT-IR, Germany) was used to characteristic the structure of MNP. A dried homogeneous sample of MNP was mixed with KBr and pressed into pellets for spectral measurement in the range 400–4000 cm^{-1}.

2.4. Scanning electron microscopy

A scanning electron microscope (SEM; FEI Quanta FEG 250 SEM, Waltham, MA,USA) was used to study the size and morphology of MNP. The sample was diluted and then a few drops were dried on a microscope slide and coated with a gold layer for examination.

2.5. In vitro studies

The Chinese hamster ovary cell line (CHO) was maintained at the National Cancer Institute (NCI), Cairo, Egypt. Cells were seeded into 96-well plates at a density of 5000 cells per well. After incubation at 37 °C in RPMI 1640 medium, 200 µL, containing 10% FBS at 37 °C in a humidified 5% CO_2 incubator, for 24 h, a portion (50 µL) of the culture medium was removed, and MNPs (final concentrations, 6.25, 12.5, 25 and 50 µg/ml) were added in PBS, 50 µL. After 30 min, cells were exposed to γ-radiation and then incubated for 72 h. Cell viability was

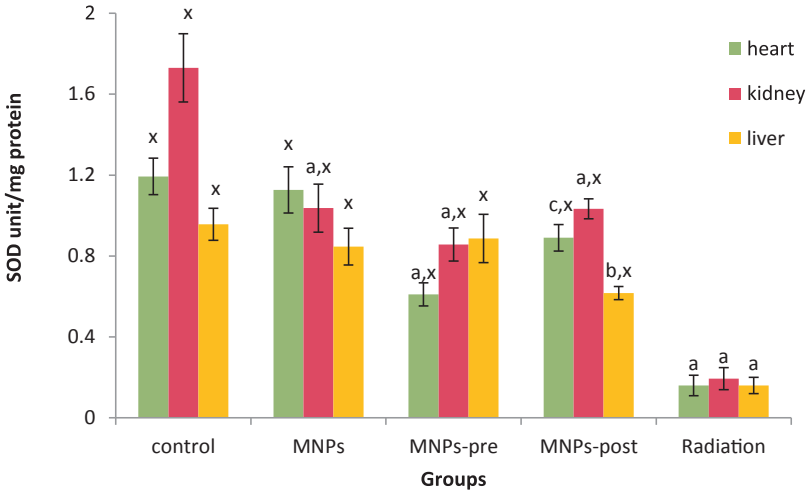

Fig. 4. Effects of MNPs pre-and post- treatment (50 mg/kg i.p.) against 7 Gy WBI on SOD activity (unit/mg protein in heart, kidney and liver tissues of mice at 4 days post irradiation. The data points are represented as mean ± SD(n = 6). (a $p \leq 0.0001$, b ≤ 0.002 and c ≤ 0.003) when compared to control group; x $p \leq 0.0001$ when compared to irradiated group.

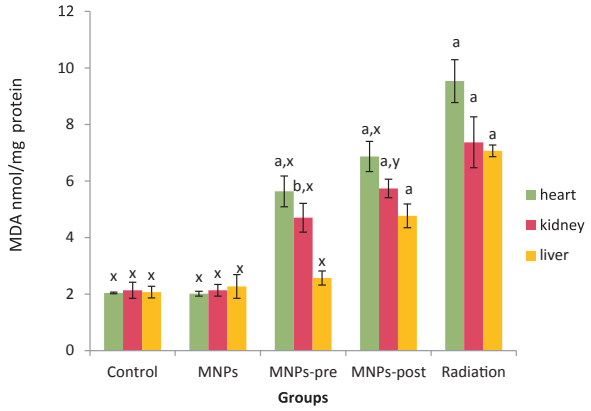

Fig. 5. Effects of MNPs pre-and post- treatment (50 mg/kg i.p.) against 7 Gy WBI on MDA level (nmol/mg protein in heart, kidney and liver tissues of mice at 4 days post irradiation. The data point are represented as mean ± SD(n = 6). (a $p \leq 0.0001$ and b ≤ 0.001) when compared to control group; x $p \leq 0.0001$ and y $p \leq 0.009$ when compared to irradiated group.

assessed with the neutral red assay [30]. Neutral red solution (20 µL) was added to each well, cells were incubated for a further 24 h, and absorbance at 540 nm was read on an Elisa micro-plate reader (Biotech, Minneapolis USA). Culture medium without MNP was used as control. Irradiation was carried out at the Middle Eastern Regional Radioisotope Centre for the Arab Countries (MERRCAC), using a ^{60}Co gamma source and a single dose of 7 Gy delivered at a rate of 0.354 Gy/min.

2.6. In vivo studies

BALB/c male mice (30 animals; 8–10 weeks old; 19.0–23.0 g), were acquired from Animal Reproduction and Artificial Insemination, Veterinary Research Division, National Research Center, Cairo, Egypt. They were accommodated in plastic cages holding autoclaved paddy covering as bedding and had access to standard mouse food and water *ad lib*. They were kept in a controlled humidity (50 ± 5%) and temperature (25 ± 3 °C) environment with 12 h light/dark cycle. The mice were divided into five groups (n = 6): control group (injected intraperitoneally (i.p.) with PBS); melanin only (injected i.p with 50 mg/kg body weight MNP); melanin pre-irradiation (injected i.p with 50 mg/kg MNP, 30 min pre- irradiation); melanin post-irradiation (injected 30 min post-irradiation), and radiation only (injected i.p with PBS and exposed to radiation).

Mice were placed in a well-ventilated container and subjected to WBI, 7 Gy. After treatment, the animals were housed under normal laboratory conditions. All groups were sacrificed on the fourth day post-irradiation. Hearts, kidneys, and livers were removed and used for evaluation. All animal procedures and care were performed according to the guidelines for the Care and Use of Laboratory Animals, Cairo University Institutional Animal Care and Use Committee (CU- IACUC), based on reviewing the application number CU/I/F/58/15.

2.7. Histopathology

Hearts, kidneys, and livers of mice were dissected, detached, fixed in 10% neutral formalin, set in paraffin blocks, divided, and stained with hematoxylin and eosin (H&E). Tissue sections were observed under an optical microscope (CX31 Olympus microscope, Tokyo, Japan) connected to a digital camera (Canon).

2.8. Comet assay (single cell gel electrophoresis)

The comet assay was performed under alkaline conditions (pH > 13) according to [31–34]. A sample (5 µL) of liver cell suspension was mixed with 0.7% low-melting-point (LMP) agarose (Sigma, A9414), 70 µL. Agarose was prepared in Ca^{2+} and Mg^{2+}-free PBS (HiMedia, TS1006) at 37 °C and placed on a microscope slide, which was already covered with a thin layer of 0.5% normal melting point (NMP) agarose (HiMedia.RM273). After cooling at 4 °C for 5 min, slides were covered with a third layer of LMP agarose. After solidification at 4 °C for 5 min, slides were immersed in freshly prepared cold lysis solution (2.5 M NaCl, 1 mM Na_2EDTA, 10 mM tris base, pH 10, with 1% Triton X-100 and 10% DMSO added just before use) at 4 °C for at least 1 h. Following lysis, slides were placed in a horizontal gel electrophoresis unit and incubated in fresh alkaline electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13). Electrophoresis was conducted for 30 min at 24 V (~0.74 V/cm) and 300 mA at 4 °C. Then, the slides were immersed in neutralizing buffer (0.4 M Tris-HCl, pH 7.5) and gently washed three times for 5 min at 4 °C. All of the above procedures were performed under dimmed light. Comets were visualized by 80??L, 1X ethidium bromide staining solution (SigmaE-8751) and examined at 400× magnification using a fluorescence microscope. Comet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera was used to measure DNA damage in 50 cells by measuring the length of DNA migration and the percentage of migrated DNA. Tail moment and Olive moment were computed [35].

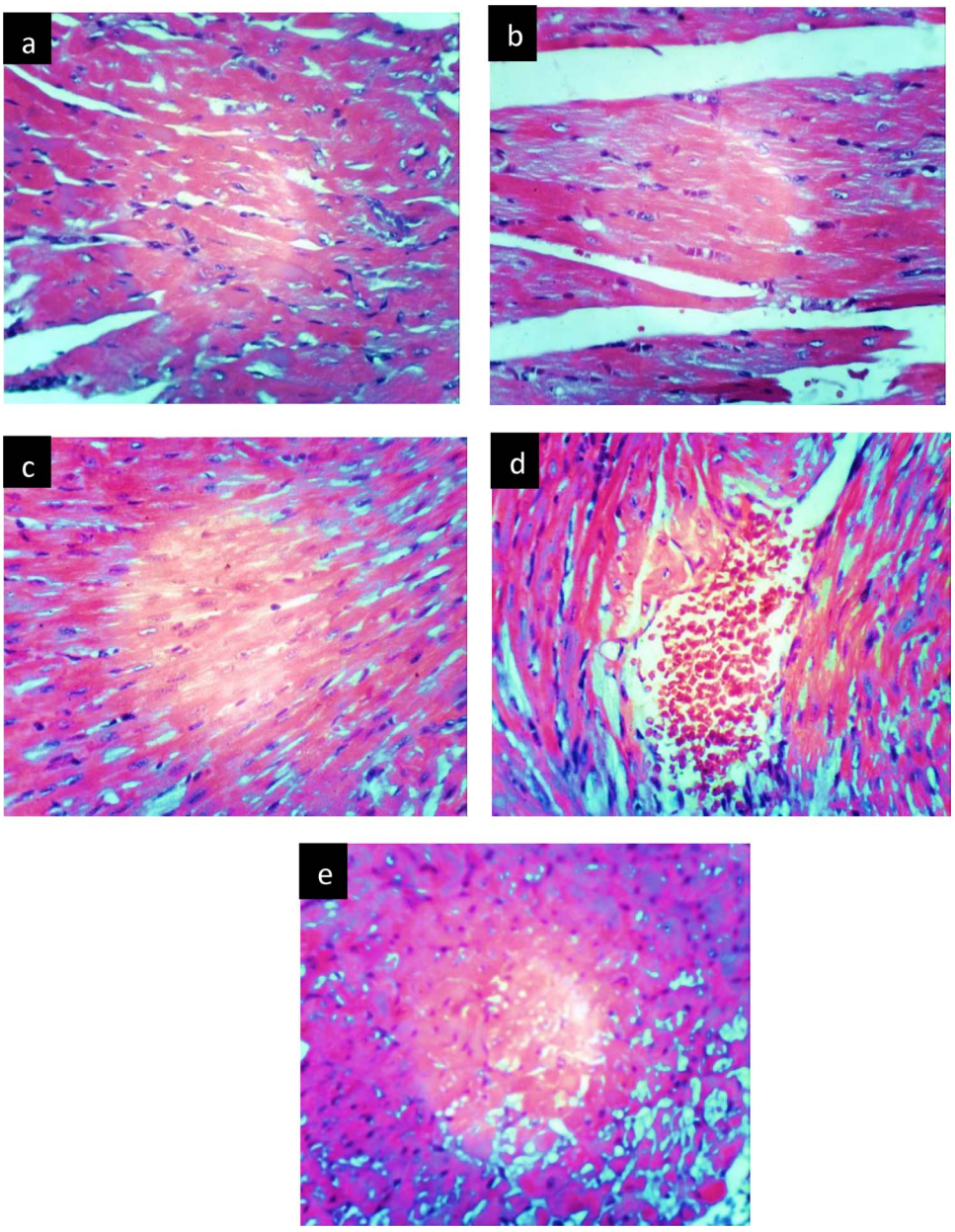

Fig. 6. Histopathological sections of heart from mice treated with MNPs (50 mg/kg i.p.) against 7 Gy WBI at 4 days post irradiation for a) control group, b) MNPs group, c) MNPs pre-irradiation group, d) MNPs post-irradiation group and e) radiation group (x400).

2.9. Biochemical estimation

Hearts, kidneys, and livers of mice from all groups were dissected and prepared for biochemical analyses. The SOD assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye measured at 560 nm [36]. Lipid peroxidation was measured by estimating the amount of malondialdehyde (MDA) according to [37]. The colorimetric assay measures the reaction of MDA with thiobarbituric acid, giving a pink complex measured at 532 nm.

3. Statistical analysis

The data were characterized as the mean ± standard deviation (SD) and were analyzed using SPSS v. 19.0 for Windows. Significant differences among groups were evaluated using one-way analysis of variance (one-way ANOVA); least-significant difference (LSD) was used for multi-groups comparisons. $P \leq 0.05$ was considered significant.

4. Results and discussion

ROS may cause damage to macromolecules, changes in intracellular oxidation-reduction reactions, metabolic stress, and cellular dysfunction. Oxidative stress causes tissue damage by promoting lipid peroxidation, DNA damage, protein modification, etc. These processes have been associated with the pathogenesis of systemic diseases of the heart, kidney, and liver [38–41].

MNP were prepared and characterized by FT-IR and SEM. The FT-IR spectrum of the MNP preparation (Fig. 1) shows a broad stretching band at about 2940–3670 cm^{-1} (N—H and O—H groups), and weak C═C, C═N, phenolic, and pyrrole ring bands at about 1639, 1424, 1023, and 737 cm^{-1} respectively [28,42,43]. MNP morphology and size were examined by SEM (Fig. 2); showing well-distributed and separated nanoparticles, diameter approx. 80 ± 5 nm.

MNP radioprotection was studied at the cellular and whole-animal levels. Irradiation reduced CHO cell viability to about 50% (Fig. 3; $P < 0.05$), in agreement with the previous work [44]. MNP

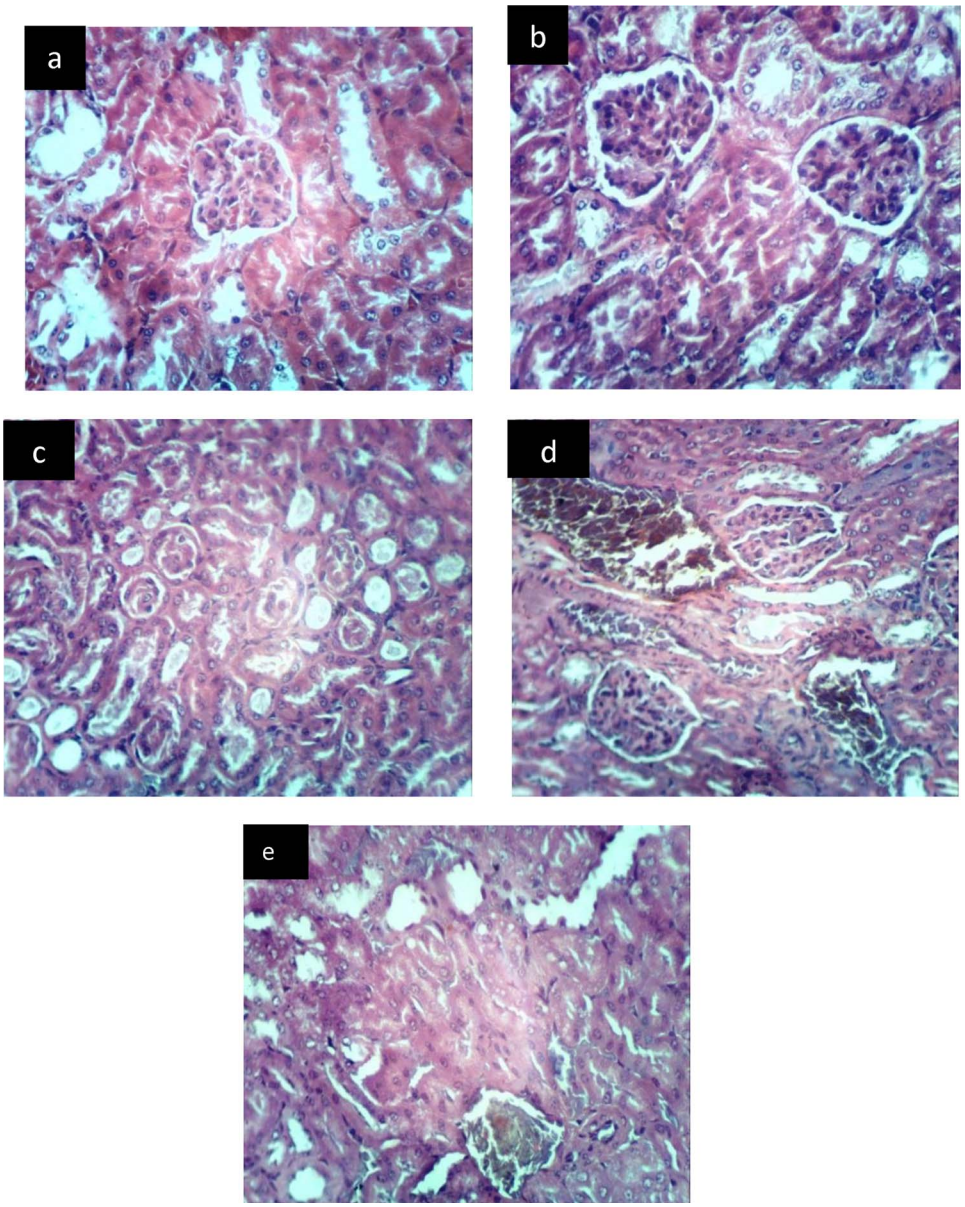

Fig. 7. Histopathological sections of kidney from mice treated with MNPs (50 mg/kg i.p.) against 7 Gy WBI at 4 days post irradiation for a) control group, b) MNPs group, c) MNPs pre-irradiation group, d) MNPs post-irradiation group and e) radiation group (×400).

pretreatment protected the cell, becoming statistically significant at 12.5 μg/ml and above, confirming that MNP can protect cells from radiation-induced cytotoxicity [45].

Measurement of antioxidant status gave different results in liver, heart, and kidney (Figs. 4 and 5). Fig. 4 shows that irradiation caused a major decrease in SOD activity ($P < 0.0001$), but this decrease was mitigated when MNP were administered either pre- or post- irradiation ($P < 0.0001$). These results are consistent with those of [46] who reported that gamma irradiation causes noticeable decrease in SOD activity in rat blood. Also, [47] observed a significant decrease in liver SOD activity in γ-irradiated mice. SOD is a first line of defense against oxygen −derived free radicals [48]. The decrease in SOD activity in the irradiated group may be a consequence of the scavenging of radiation-induced ROS.

The effects of MNP treatment and/or irradiation on lipid peroxidation, measured as MDA, in liver, heart, and kidney tissues, are shown in Fig. 5. Irradiated mice showed elevation ($p < 0.0001$) of MDA levels by approximately 3- to 5-fold compared to controls. These results are in agreement with [49–51]. Radiation-induced lipid peroxidation was also inhibited by pre –or post-administration of MNP, by approximately 1- to 3-fold. Elevated levels of lipid peroxidation have been linked to injurious effects such as loss of fluidity, inactivation of membrane enzymes, increases in permeability to ions, and eventually disruption of cell membrane integrity leading to release of cell organelles [52–54]. These results are reflected in the histopathological changes in heart, kidney, and liver tissues (Figs. 6–8). As shown in Fig. 6e, vacuolization of the sarcoplasm of cardiac myocytes was observed in heart tissue of the irradiated group compared with the control and melanin groups (Fig. 6a and b), which show normal cardiac myocytes. Treatment with MNP protected the cardiac tissue (Fig. 6c and d).

Kidneys of mice from the control and melanin groups did not show histopathological changes when compared to normal parenchyma (Fig. 7a and b). However, radiation caused alterations (Fig. 7e), including renal blood vessel damage and fatty change of the epithelial lining of the renal tubules. MNP treatment pre- or post- irradiation provided some protection (Fig. 7c and d), as observed by epithelial cast in the lumen of the renal tubules and congestion in the blood vessels, respectively. The hepatic histology of the irradiated group demonstrates

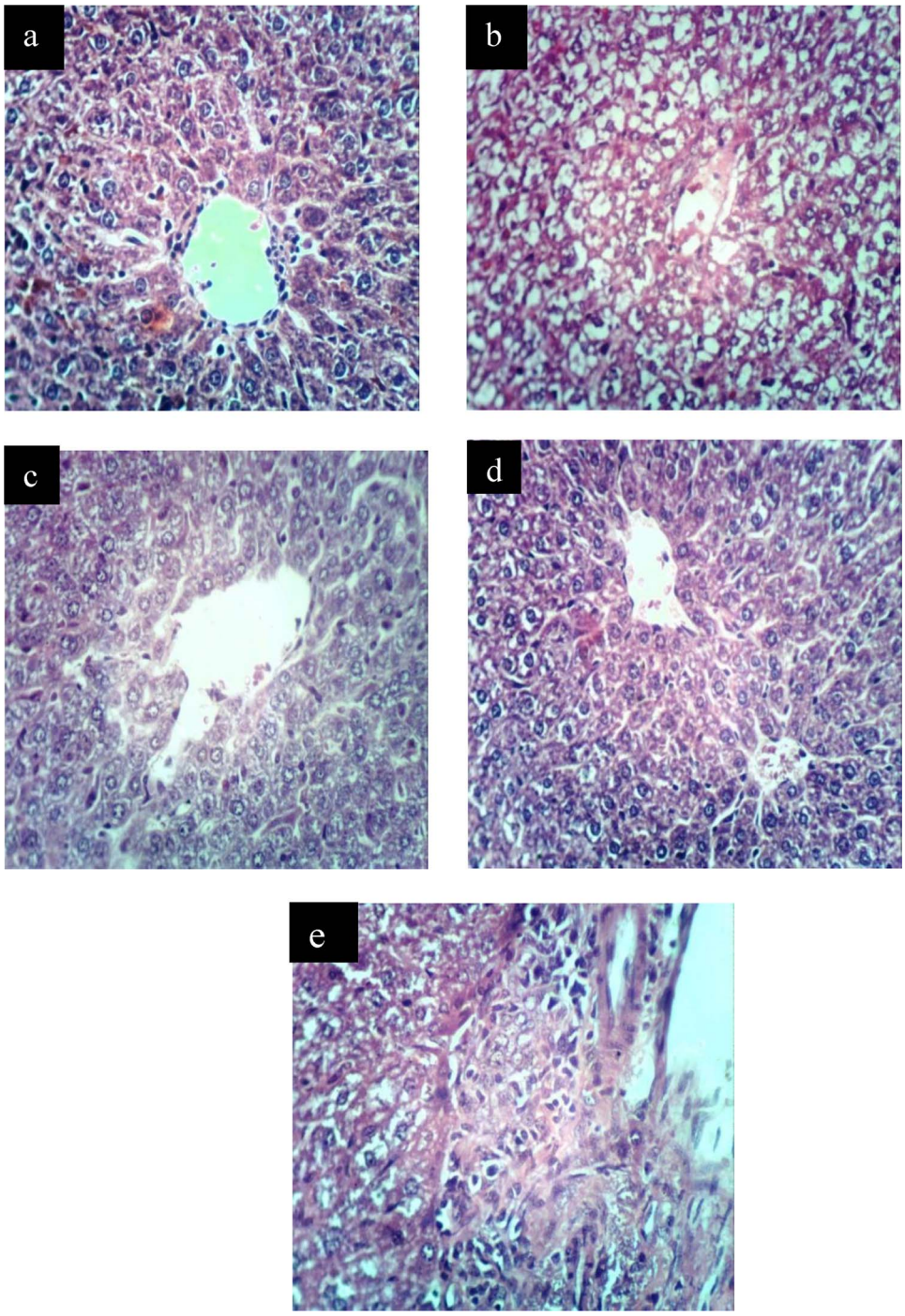

Fig. 8. Histopathological sections of liver from mice treated with MNPs (50 mg/kg i.p.) against 7 Gy WBI at 4 days post irradiation for a) control group, b) MNPs group, c) MNPs pre-irradiation group, d) MNPs post-irradiation group and e) radiation group (x400).

partial fibrosis with infiltration and appearance of inflammatory cells (Fig. 8e). MNP pre- and post-treatment showed improvement in hepatocyte morphology and fewer inflammatory cells, e.g. Kupffer cells (Fig. 8c and d). The hepatocytes appeared normal in the control group (Fig. 8a), and cytoplasmic vacuolization in the melanin group was also normal (Fig. 8b).

MNP protection against radiation-induced liver DNA-damage at the fourth day post- irradiation was studied with the comet assay [10,34]. In the irradiated group, comet parameters showed significant increases ($P \leq 0.0001$) compared with control (Fig. 9a–d). MNP protected the DNA. The melanin group showed nearly the same results as the control group, confirming the lack of genotoxicity of MNP. Ionizing radiation-induced DNA damage is usually repaired within hours, post-irradiation. Here, DNA damage was still detected four days after irradiation, in agreement with [55]; Vijay et al., 2012 and [24]. The explanation might be the imbalance between oxidative damage and the antioxidants system caused by ionizing radiation [56]; Tomomi et al., 2016; Ifigeneia et al., 2017.

Exposure to 7 Gy ionizing radiation induces ROS, also seen as an increase in oxidized lipids (MDA). Some ROS are detoxified by SOD; some of the available SOD is used up and global SOD activity is decreased. The increase of MDA and decrease of SOD levels are concomitant with induction of DNA damage. Generally, free radicals and ROS induced by radiation are toxic to cells [57–59]. MNP, expected to

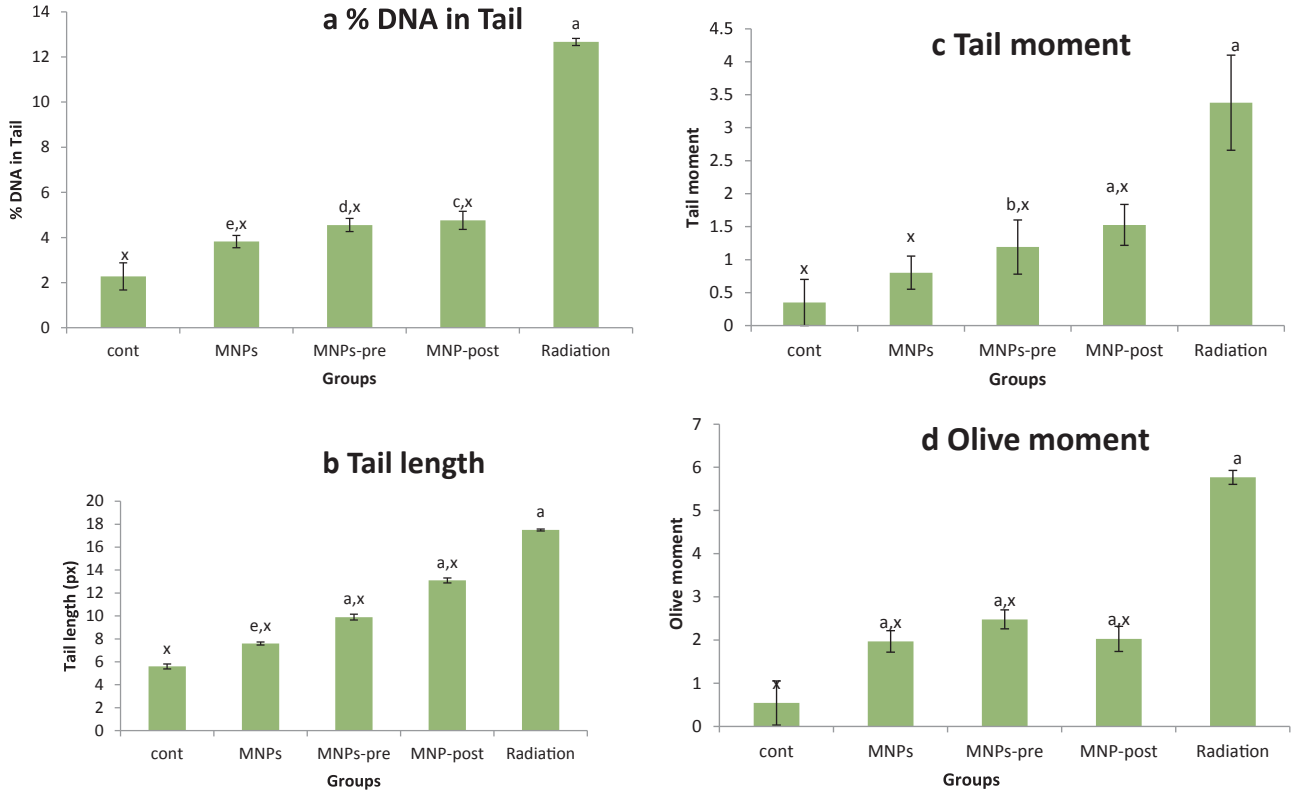

Fig. 9. Modulation of comet parameters by MNPs (50 mg/kg; i.p.) pre- and post-treatment against 7 Gy WBI-induced DNA damage in liver cells a) percentage DNA in tail, b) tail length, c) tail moment and d) olive moment at 4 days post-irradiation.The data point are represented as means ± SD (n = 6). (a $p \leq 0.0001$, b $p \leq 0.001$,c $p \leq 0.02$, c $p \leq 0.04$ and d $p \leq 0.05$) when compared to control group; x $p \leq 0.0001$ when compared to irradiated group.

act as antioxidant and antiradical agents, exhibit protective effects, as seen in the present study, by partial restoration of SOD activity and reduction of MDA levels after irradiation.

5. Conclusions

MNPs can protect mice against oxidative stress and DNA damage induced by ionizing radiation. MNP appear not to produce tissue damage and may be considered as harmless antioxidant and antiradical compounds with immuno-modulatory properties. Moreover, MNP treatment after irradiation may be useful to prevent side effects on tumor-adjacent tissues caused by long-lived radicals and oxidative stress in cancer radiation therapy.

Conflict of interest

The authors state that there are no conflicts of interest.

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