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Therapeutic Effect of Melatonin and/or Adipose-Derived Mesenchymal Stem Cells Against Doxorubicin-Induced Renal Toxicity in Wister Rats

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Abstract:

Background: Doxorubicin (DOX) is highly effective against cancer, but its use is limited due to significant toxicity, particularly in the liver and kidneys. This toxicity is primarily caused by oxidative stress, which results in biochemical, histological, and genetic abnormalities, and disrupts the body's antioxidant defenses. Adipose-derived mesenchymal stem cells (ADMSCs) show promise in cell therapy applications against DOX toxicity Similarly, melatonin supplementation has demonstrated therapeutic potential in mitigating DOX-induced liver and kidney damage by reducing oxidative stress. Understanding and harnessing these therapeutic effects could lead to improved treatments for DOX-induced organ damage.

Aims: This study focused on evaluating how melatonin and adipose-derived mesenchymal stem cells (ADMSCs) could mitigate renal toxicity induced by doxorubicin (DOX), a potent anthracycline chemotherapeutic drug known for its severe side effects. The research aimed to explore whether the antioxidant properties of melatonin and the regenerative capabilities of ADMSCs could synergistically alleviate the adverse effects of DOX on renal function in Wistar rats.

Methods: The study included two main groups: a control group receiving saline and a DOX group receiving six doses of doxorubicin to induce renal toxicity. Subsequently, rats from both groups were assigned to receive treatments of saline, ADMSCs,

melatonin, or a combination of ADMSCs and melatonin. Rats were euthanized at different time points post-treatment, and blood and kidney tissue samples were collected. The analysis included assessment of oxidative stress biomarkers, DNA damage, gene expression profiles, histopathological changes, and cell homing in the kidney tissues.

Results: DOX induces oxidative stress, inflammation, and genotoxicity in kidneys while reducing antioxidant enzyme activity. Melatonin and/or ADMSC treatments effectively mitigate these effects by decreasing oxidative stress, inflammation, and DNA damage. The protective mechanisms involve modulation of apoptosis pathways, evidenced by changes in p53 and Bcl2 expression levels, as well as assessment of double-strand breaks using the neutral comet assay, and also the improvement of the inflammatory markers IL-6 & IL-10 evidenced by the histopathological results.

Conclusion: Melatonin and ADMSCs demonstrate protective effects against DOX-induced renal toxicity, potentially enhancing chemotherapy safety by reducing renal damage. The synergistic benefits of combining melatonin and ADMSC therapy in improving chemotherapy tolerability warrant further investigation in preclinical models to optimize treatment strategies and validate efficacy before clinical application.

Keywords: Doxorubicin; Melatonin; adipose-derived mesenchymal stem cells (ADMSC); Genotoxicity; apoptosis; inflammation; Renal toxicity.

1. Introduction

Doxorubicin (DOX), an anthracycline chemotherapeutic agent, inhibits topo-isomerase-2 enzyme to impede cancer cell proliferation (Kariuki et al., 2024). Widely used for breast, ovarian, lung, bladder cancers, leukemia, AIDS-related Kaposi's sarcoma, and solid tumors (Alghorabi et al., 2019), it lacks specificity, causing significant side effects such as cardiotoxicity, neuropathy, hepatotoxicity, nephrotoxicity, alopecia, typhlitis, myelosuppression, neutropenia, anemia, thrombocytopenia, nausea, and diarrhea (Ajaykumar, 2021b). DOX causes nephropathy by interfering with kidney podocytes, leading to proteinuria and structural changes in nephrons, including glomerulosclerosis and tubular dilation (Ajaykumar, 2021b; Lahoti et al., 2012). Earlier studies indicate that oxidative stress, inflammatory response, and cell apoptosis underlie the toxicity of DOX. It was reported that DOX caused a severe reduction in SOD activity and GSH levels, accompanied by an increase in MDA, leading to the overproduction of ROS and induction of oxidative injuries (Wu et Al., 2021). It induces mitochondrial damage in renal cells via reactive oxygen species (ROS) from mitochondrial DNA interference and forms ROS-producing iron complexes, exacerbating oxidative stress and depleting antioxidant levels like glutathione and vitamin E (Lebrecht et al., 2003; Wang et al., 2000; Rook et al., 2004). This oxidative damage is linked to nephron deterioration, hypertension, renal failure, and metabolic disturbances (Fogo, 2007). Angiotensin-converting enzyme activity is implicated in the tissue damage caused by DOX therapy (Rook et al., 2004). To mitigate the acute toxic side effects of cancer chemotherapy on organs like the liver and kidney, combining drug delivery methods with potent antioxidant agents has been proposed as an effective strategy (Lee et al., 2011, Wu et al., 2021). This approach aims to reduce chemotherapy-related toxicity while preserving the drugs' therapeutic efficacy. Nephrotoxicity affects approximately one-third of chemotherapy patients, leading to tubular cell death (El-Sayyad et al., 2016). In Egypt, renal failure is prevalent due to cancer treatment, drug misuse, and environmental toxins. Mesenchymal stem cell therapy is proposed as an alternative to renal transplantation, addressing cost, immunosuppressive drug dependency, and organ rejection concerns (El-Sayyad et al., 2016).

Mesenchymal stem cells (MSCs) are readily expandable and sourced from medical waste like adipose and umbilical cord tissues. They exhibit properties influenced by culturing conditions and demonstrate anti-inflammatory, antioxidant, anti-fibrotic, and angiogenic effects (Kojima et

al., 2019). Adipose-derived mesenchymal stem cells (ADMSCs) offer advantages in regenerative medicine due to their easy acquisition from adipose tissue, large-scale expansion capability, and differentiation into osteoblasts, adipocytes, and chondrocytes via key transcription factors. They exhibit therapeutic efficacy in treating atrophy, fibrosis, ulcers, and wound healing, alongside immunomodulatory effects beneficial for hematological and immunological diseases. ADMSCs thus hold substantial promise in various regenerative applications (Heo et al., 2019).

Melatonin, synthesized from tryptophan in the pineal gland, regulates circadian rhythms and exhibits antioxidant, anti-inflammatory, and cell proliferation control properties. It enhances the effectiveness of anticancer drugs like DOX, cisplatin, epirubicin, and bleomycin by modulating various signaling pathways, potentially allowing for reduced therapeutic doses and mitigating the adverse effects of chemotherapy. Melatonin's synergy with doxorubicin notably promotes cancer cell apoptosis (Tran et al., 2021). Melatonin demonstrates diverse physiological functions including anti-inflammatory, antioxidant, anti-apoptotic, autophagy-regulating, and potential antitumor effects. It has been shown to exert cyto-protective effects against ischemic injuries in organs such as the heart, brain, liver, and kidney (Han et al., 2015b).

Melatonin enhances the functionality of ADMSCs by acting through its receptors. It improves ADMSC proliferation, differentiation into osteogenic lineages, and immunomodulatory capabilities. Studies using melatonin antagonists confirm its role in enhancing ADMSC performance, viability and osteogenic differentiation potential (Heo et al., 2019; Skubis-Sikora et al., 2023). Han et al. (2015) investigated melatonin's impact on AD-MSCs in myocardial infarction (MI) injury. They found that melatonin induces overexpression of antioxidant enzymes like catalase and superoxide dismutase-1 in MSCs, enhancing their resistance to hydrogen peroxide-induced apoptosis. Melatonin's anti-inflammatory and antioxidant properties were evaluated for their ability to support AD-MSCs' survival and cardio-protective effects in vivo, ultimately improving cardiac function post-MI. Melatonin enhances the survival and functional efficacy of AD-MSCs in infarcted hearts, synergistically restoring heart function. In vivo, melatonin reduces inflammation, apoptosis, and oxidative stress in infarcted heart tissue. In vitro studies demonstrate melatonin's cyto-protective effects on AD-MSCs under hypoxia and serum deprivation conditions by mitigating inflammation, apoptosis, and oxidative stress. These findings suggest melatonin as a promising adjunct to improve MSC-based therapies for heart repair (Han et al., 2015).

Melatonin shows promise in enhancing ADMSCs by improving their proliferation, differentiation, and immunomodulatory functions, as demonstrated in vitro. Although the exact mechanisms are not fully elucidated, these findings suggest melatonin as a novel strategy to enhance the outcomes of cell therapy (Heo et al., 2019).

This study investigates how melatonin synergistically enhances the viability of ADMSCs and mitigates liver injury caused by DOX. It focuses on reducing oxidative stress and inflammation associated with DOX administration while exploring mechanisms to enhance DOX's therapeutic efficacy and minimize liver-related adverse effects. These findings provide critical insights into developing advanced adjunct therapies that could significantly improve the safety and effectiveness of cancer treatment protocols.

Materials and Methods

Ethics:

This study complied with ARRIVE guidelines and was approved by the Cairo University Institutional Animal Care and Use Committee (CU-IACUC), Egypt, number CU/I/F/42/20. All procedures are carried out in strict accordance with international standards of animal care and use to ensure ethical conduct and compliance with legal requirements.

Chemicals:

DOX was obtained from Ebewe Pharma in Egypt, while Melatonin (5 mg) was sourced from Nature's Bounty. Adipose-derived mesenchymal stem cells (AdMSCs) were provided by Prof. Dr. Laila Rashid from Kasr El Ainy in Giza, Egypt. The quantitative real-time polymerase chain reaction (RT-PCR) kits were purchased from Thermo Fisher Scientific, Inc., in the USA and Sigma-Aldrich Co., also in the USA. All chemicals were of high purity and purchased from well-known international suppliers.

Animal Model and Housing Conditions:

This study was conducted using 32 male Wistar rats, weighing approximately 170 ± 10 g each, obtained from the National Research Institute, Dokki, Giza, Egypt. These animals were housed in groups of five per cage in a controlled environment at the Animal Center of the Department of Zoology, Cairo University. A 12-hour light/dark was used in the facility and the temperature was maintained at 25 ± 2°C. The rats were provided with unrestricted access to water and a standard pelleted diet containing 20% protein. They underwent a one-week acclimatization period before the commencement of the experiment.

Experimental Design:

At the beginning of the study, the animals were divided into two main groups: a negative control group with 15 rats and a doxorubicin (DOX) positive control group with 47 rats. The experiment spanned a total of thirty-four days, which included a 14-day induction phase followed by a 20-day therapeutic phase.

During the induction phase, the negative control group (CI) received saline as a vehicle for DOX and continued their regular diet. In contrast, the DOX group (DI) received six doses of doxorubicin (2.5 mg/kg, administered intraperitoneally every other day to induce hepatotoxicity. After this phase, five rats from each group were euthanized for initial analysis, and the remaining rats were divided into five distinct subgroups for the therapeutic phase. These subgroups included a negative control subgroup (CT) continuing to receive saline treatment, a positive control subgroup (DT) receiving the DOX vehicle, and three treatment subgroups: one receiving a single intravenous dose of adipose-derived mesenchymal stem cells (ADMSCs) (1 x 106 cells in 0.5 ml DEMEM per rat) (SCT), one treated with melatonin at a dosage of 10 mg/kg administered intraperitoneally every other day throughout the therapeutic phase (MT), and one receiving a combination of ADMSCs and melatonin (SCMT).

The homing of labeled stem cells was monitored 24 hours after transplantation in stem cell groups, with additional assessments conducted midway through and at the end of the study phase. These assessments involved euthanizing rats to perform thorough analyses of kidney tissues at physiological, biochemical, molecular, and histological levels.

Blood Collection and Tissue Processing:

After each experimental phase, five rats per group were anesthetized with sodium pentobarbital (100 mg/kg) and euthanized by cervical dislocation. Blood samples were collected from the retro-orbital plexus and centrifuged at 3000 rpm for 10 minutes to obtain serum after clotting. The kidneys were excised, washed with saline, dried gently, and weighed. Each kidney was then divided into two halves: one half was preserved in 10% formalin for histopathological examination and tissue homing studies, while the other half was homogenized in 10% (w/v) phosphate-buffered saline (PBS) and stored at −80°C for molecular analyses.

Somatic indices:

Initial and final body weights, along with hepatic and renal indices, were documented throughout the experiment.

Biochemical Assessments:

oxidative stress markers were measured in kidney including catalase (CAT), malondialdehyde (MDA), nitric oxide (NO), glutathione (GSH), and superoxide dismutase (SOD) were assessed using spectrophotometric methods. CAT activity was determined following Aebi's method, and MDA levels were quantified as per Ohkawa et al.'s procedure [Ohkawa et al., 1979]. Liver tissues were homogenized in 0.1M Tris-HCl (pH 7.4) and then centrifuged at 25,000 rpm for 10 minutes at 4°C. The resulting supernatant was analyzed for MDA, NO, GSH, SOD, and CAT levels using specific commercial kits from Biodiagnostics in Egypt, following the manufacturer's instructions and established protocols.

Quantitative Real-Time PCR (qRT-PCR) Methodology:

or quantitative real-time PCR (qRT-PCR), total RNA was extracted from rat kidney homogenates using the RNeasy Mini kit from Qiagen, following the manufacturer's instructions. The concentration of RNA was determined using a Nanodrop spectrophotometer from Thermo Scientific. Subsequently, 1 µg of total RNA was reverse transcribed into cDNA using the RevertAid cDNA Synthesis Kit from Thermo Fisher Scientific. The expression levels of apoptotic genes (p53, Bcl2) and inflammatory genes (IL-6, IL-10) were analyzed with GAPDH serving as a reference gene. The qPCR reactions were performed using the HERAPLUS SYBR® Green qPCR Kit from Willowfort on an Applied Biosystems Step One Plus system. Primers were sourced from Macrogen, Inc. (Seoul, Korea) as detailed in Table 1. The qPCR protocol included an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, concluding with a final extension at 72°C for 10 minutes. Specificity of the amplification was confirmed through melting curve analysis. Data were analyzed using the ΔΔCt method to calculate relative expression levels (RQ) and fold changes.

Table 1: Primer sequences of genes involved in RT-qPCR.

Gene	Primer sequence	
p53	Forward	5'-CTGAAGACTGGATAACTGTC-3'
	Reverse	5'-CTTATTGAGGGAAATTGAGT-3'
BCL 2	Forward	5'-GGTGGACAACATCGCTCTG-3'
	Reverse	5'AGACAGCCAGGAGAAATCAAAC-3'
IL-6	Forward	5’-GCCCTTCAGGAACAGCTATGA-3’
	Reverse	5’-TGTCAACAACATCAGTCCCAAGA-3’
IL-10	Forward	5’-GCAGGACTTTAAGGGTTACTTGG-3’
	Reverse	5’-CCTTTGTCTTGGAGCTTATTAAA-3’

Comet assay (alkaline and neutral):

The Comet Assay (alkaline and neutral) was used for assessment of DNA damage within individual cells. Slides were prepared using Normal Melting Agarose (NMA) and Low Melting Point Agarose (LMPA) obtained from Sigma Chemical Company, along with other required reagents. Following the methodology described by Singh et al. (2003) with minor modifications, kidney tissues were homogenized in PBS, mixed with LMPA, and applied onto NMA-coated slides. After lysing in a specific solution, slides underwent electrophoresis in an alkaline buffer to unwind DNA strands, followed by neutralization and drying. The neutral Comet Assay variant was conducted according to the protocol by Boutet-Robinet et al. [Boutet-Robinet et al., 2013].

Ethidium bromide staining was used for fluorescence microscopy to analyze DNA fragmentation, assessing parameters such as tail length, DNA percentage in the tail, tail moment, and olive tail moment. This method provided a detailed quantitative evaluation of DNA damage and enabled us to investigate the protective effects of our treatments against doxorubicin-induced renal toxicity.

Homing of labeled stem cells by fluorescence microscope in kidney tissues:

The homing of ADMSCs was studied by tracking cells labeled with PKH26, a red fluorescent dye. Two Wistar rats previously treated with stem cells received an intravenous injection of 1 x 106 PKH26-labeled ADMSCs via the tail vein. After 48 hours, the rats were euthanized, and kidney tissues were collected for analysis. Fluorescent microscopy was employed to examine the tissues, allowing for the detection and evaluation of stem cell migration to the affected organs. This approach assessed the effectiveness of the administered ADMSCs in homing to the damaged tissues.

Histopathological Examinations of Liver Tissues:

Comprehensive histopathological assessments were performed to evaluate the structural integrity of hepatic tissues post-treatment. After euthanasia, kidney samples from each treatment group were fixed in 10% buffered formalin, sequentially dehydrated in ethanol solutions, cleared in xylene, and embedded in paraffin. Tissue sections, cut at a thickness of 5 μm, were stained with hematoxylin and eosin to highlight any histological changes. These sections were examined under a light microscope (Olympus BX50, Japan), following the methodology described by Bancroft & Stevens [Bancroft & Stevens, 1996], to analyze the therapeutic effects of the treatments on tissue architecture.

Statistical Analysis:

Statistical analysis was performed using SPSS software, version 25. The data were initially subjected to one-way ANOVA to compare group means, followed by Duncan's post hoc test for specific pairwise comparisons. Statistical significance was set at $P \leq 0.05$. Results are expressed as mean values accompanied by standard errors (mean ± SE).

Results:

Body Weight, liver Weight, and renal Somatic Index:

The total body weight, kidney weight and renal somatic index (RSI) of all groups are presented (Figure 1). During the induction phase, the DI-treated group showed significant reductions in the total body weight and kidney weight, as compared to the CI group. However, no significant difference was observed in the RSI between the CI- and DI-treated groups.

After 10 and 20 days of treatment, as compared to the CT group, the total body weight and kidney weight were markedly reduced in most of the remaining groups. However, no marked changes in the RSI were reported in any of the experimental groups, as compared to the CT group, after 10 and 20 days of treatment.

Oxidative Stress Biomarkers:

The activity of CAT, SOD and levels of GSH, MDA and NO in the renal tissue of all groups are reported (Figs 2-4).

During the induction phase, the DI-treated group showed significant elevations in the MDA and NO levels were associated with marked reductions in the activities of CAT and SOD as well as the GSH content, as compared to the CI-group. Rats of DT-group showed marked declines in CAT activity (-55%), SOD activity (-80%) and GSH content (-69%) whereas marked elevations in the levels of MDA (+153%) and NO (+104%), as compared to the CT -group. In the DT-group, marked declines in CAT activity by -55%, as compared to the CT -group. However, SCT, MT and SCMT groups showed marked elevations in the CAT activity by +90%, +78% and +116%, respectively, as compared to the DT-treated group.

In comparison to the CI-group, SCT, MT and SCMT groups showed significant (P=0.000) declines in the activities of CAT, SOD and level of GSH except for insignificant changes in the SCMT group. In addition, all the experimental groups showed marked elevation in the MDA and NO levels except for insignificant change in NO level in SCT-group and MDA level in SCMT-group, as compared to the CT group.

As compared to the DT-group, SCT, MT and SCMT groups showed marked elevations in the activities of CAT (+90, +78 & +116%), SOD (+161, +127 & +398%), and level of GSH (+112, +40 & +227%), respectively. However, in comparison of DT-group, the MDA and NO levels in SCT, MT and SCMT groups showed marked reductions in the levels of MDA (-48, -30 & -65%), and NO (-49, -38 & -65%), respectively.

Gene Expression Analysis of P53, Bcl-2, IL-6 and IL-10:

The gene expression levels of P53, Bcl-2, IL-6, and IL-10 in the renal tissue of all groups are displayed (Figs 5-6).

During the induction phase, the DI-treated group showed significant upregulations in the gene expression level of P53 and IL-6 whereas marked downregulations in the mRNA levels of Bcl-2 and IL-10, as compared to the corresponding control group (CI-group).

In the treatment phase, DT-treated group showed marked elevations in the gene expression of P53 and IL-6 by +220% and +354%, as compared to the CT-group, respectively. The mRNA levels of P53, in the SCT, MT and SCMT groups were downregulated by -77%, -73% and -74%, respectively, as compared to the DT-treated group. The gene expression level of IL-6 showed downregulation in SCT, MT and SCMT groups by -90%, -86% and -86%, respectively, as compared to the DT-treated group. In the DT-treated group, Bcl-2 and IL-10 gene expression levels were significantly (P=0.000) lower than the CT group. As compared to the DT-group, Bcl-2 and IL-10 gene expression levels showed marked upregulations in the SCT (+632%, +681%), MT (+570%, +630%) and SCMT (+577%, +664%), respectively.

DNA Damage Assessment Using Alkaline and Neutral Comet Assay:

The results of alkaline and neutral comet parameters, tail length (TL), DNA damage in tail%, tail moment (TM), and olive tail moment (OTM) in the kidney are presented. The results of alkaline and neutral comet parameters in the kidney are displayed (Figures 7-10). During the induction and treatment phases, significant (P=0.000) elevations in all the studied alkaline and neutral comet parameters were recorded in the kidney of the DI-group and DT-group, as compared to the corresponding control groups.

According to the alkaline comet technique, SCT-, MT- and SCMT-groups showed remarkable declines in all the studied comet parameters in the kidney, as compared to the DT-group but were insignificantly different from the control group.

According to the neutral comet technique, SCT-, MT- and SCMT-groups showed remarkable declines in all the studied comet parameters in the kidney, as compared to the DT-group. Moreover, most of the studied comet parameters in the SCT-, MT- and SCMT groups were remarkably greater than the control group.

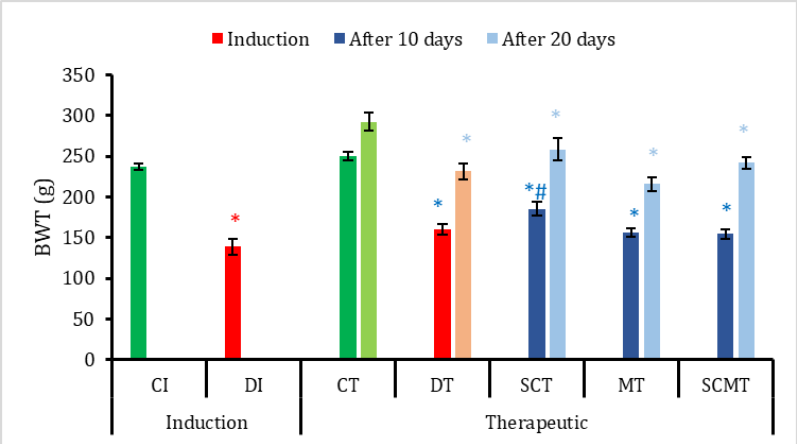
Histopathological Examination:

In our investigation, Kidneys histopathological results revealed different histological lesions among the different experimental groups (Figure 11). In control groups (CI & CT) showed normal renal parenchyma with normal glomeruli, normal renal tubules, and normal interstitial tissue.

In contrast, the DOX-treated group (DI) revealed glomerular degeneration and atrophy with mononuclear cell infiltration in the interstitial tissue. The group receiving DOX therapy (DT) further exhibited diffuse renal tubule degeneration and necrosis. Remarkably, the stem cell treatment group (SCT) noted congestion in the interstitial blood vessel and cellular cast inside the renal tubules. The melatonin-treated group (MT) showed cellular cast inside the renal tubules, there were no signs of necrosis or congestion. Most notably, the combination treatment group (SCMT) mirrored the healthy renal architecture seen in control groups; normal renal parenchyma with neither sign of congestion nor fibrosis nor necrosis, demonstrating the synergistic potential of stem cells and melatonin in improving DOX-induced toxic effects.

MSCs Homing to the Heart:

In vivo fluorescence imaging provided supplementary evidence of MSCs homing. MSCs, labeled with red PKH26 and delivered intravenously via the tail vein, were observed in kidney tissue within two days after injection. This migration is illustrated in Figure 12.



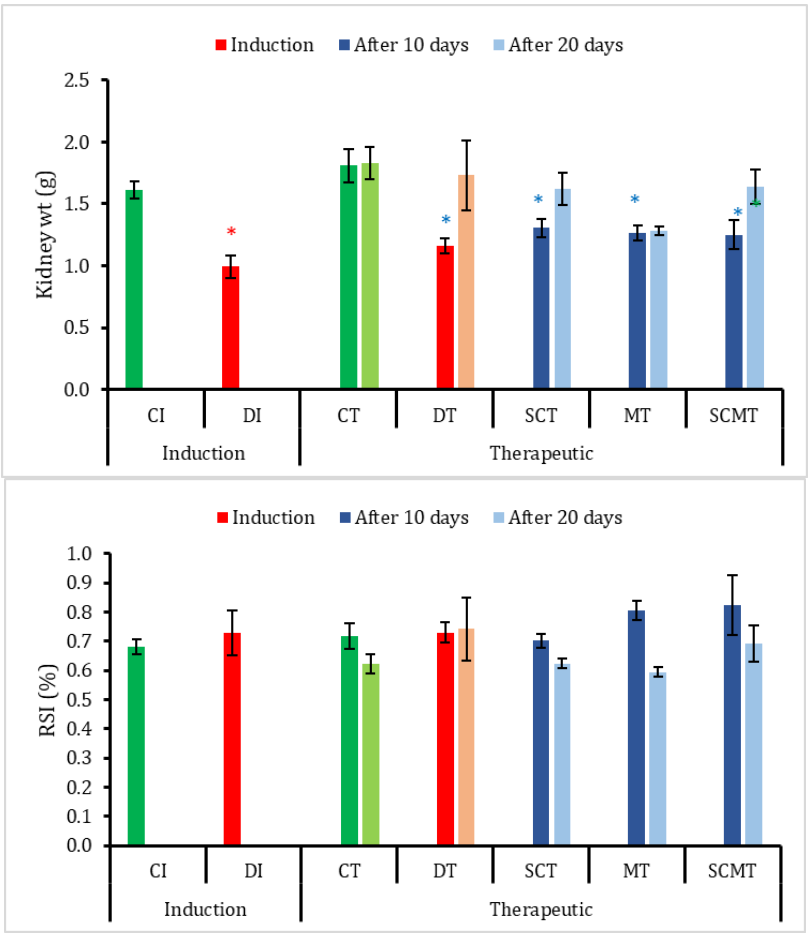


Fig 1. The total body weight, kidney and renal somatic index (RSI) in all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

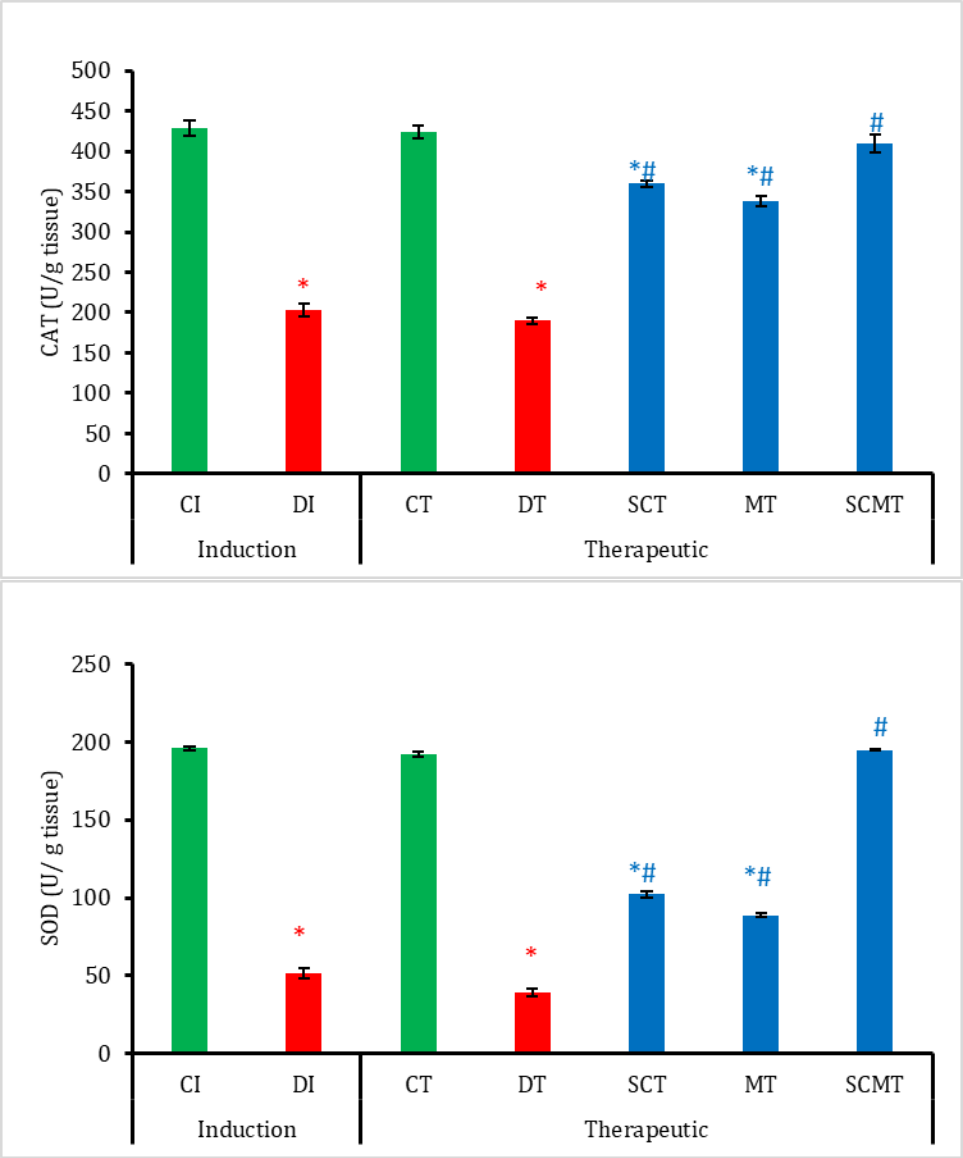


Fig 2. The activity of CAT and SOD in the kidney of all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

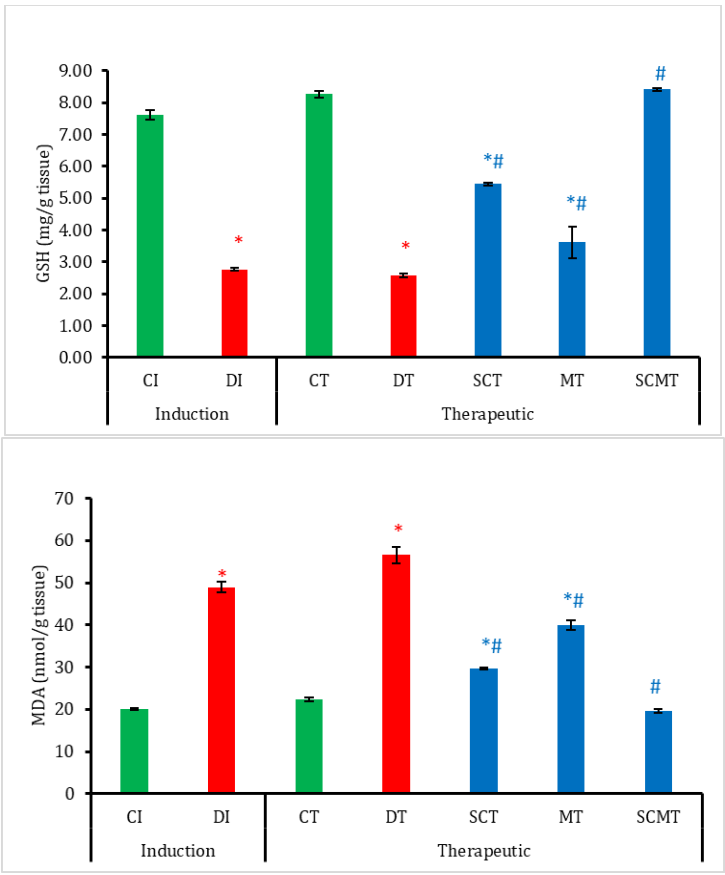


Fig 3. The level of GSH and MDA in the kidney of all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

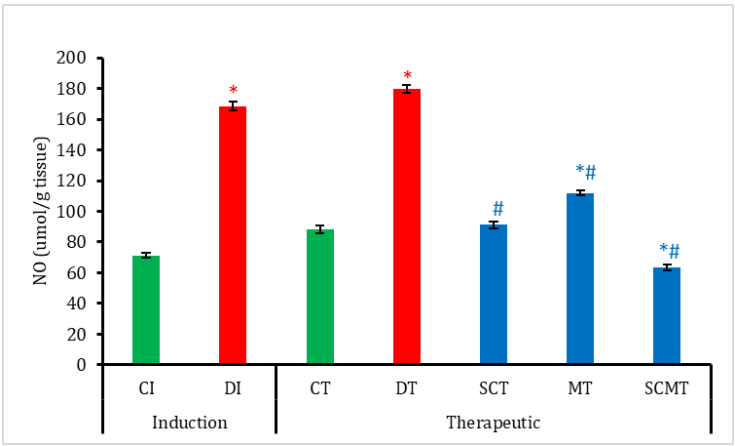


Fig 4. The level of NO in the kidneys of all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

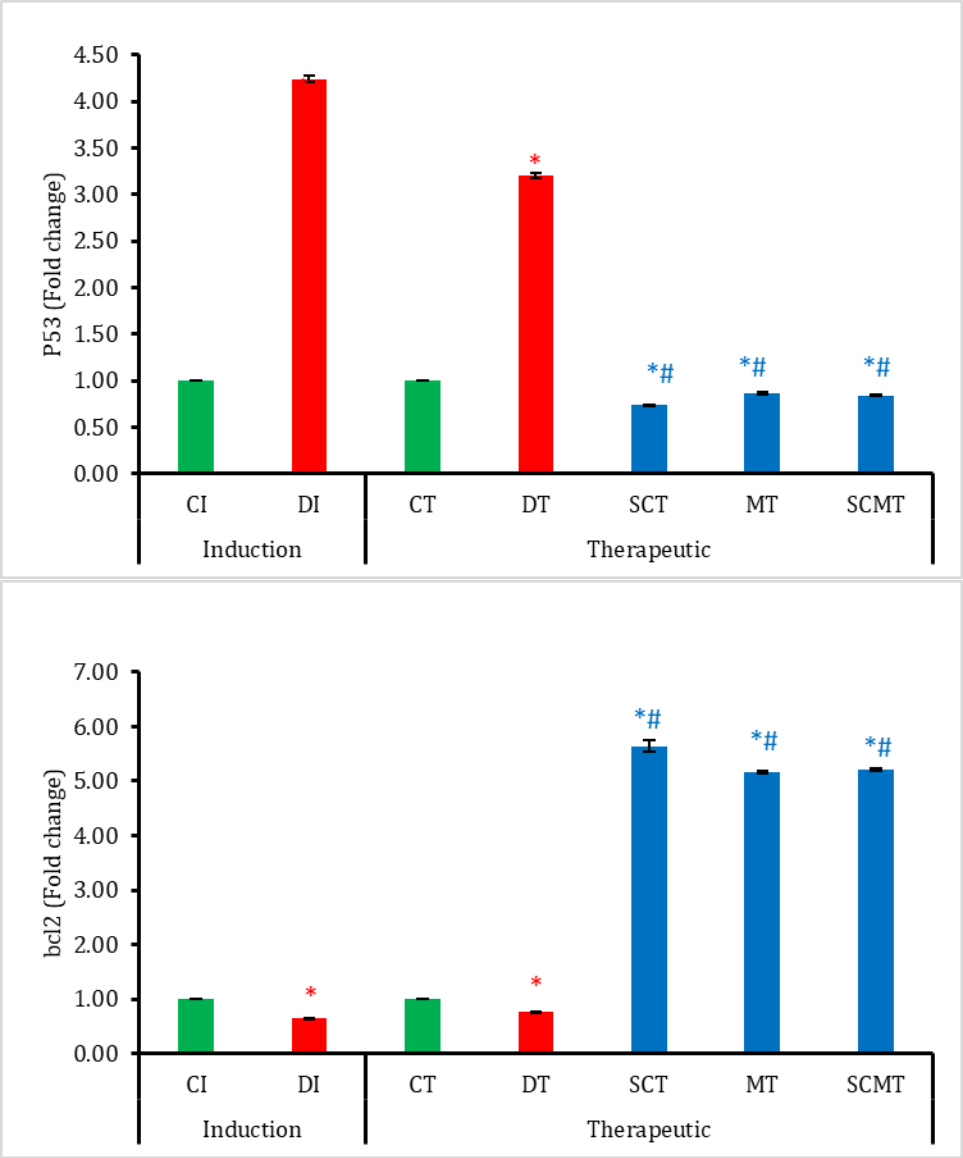


Fig 5. The gene expression levels of p53 and bcl-2 in the liver of all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

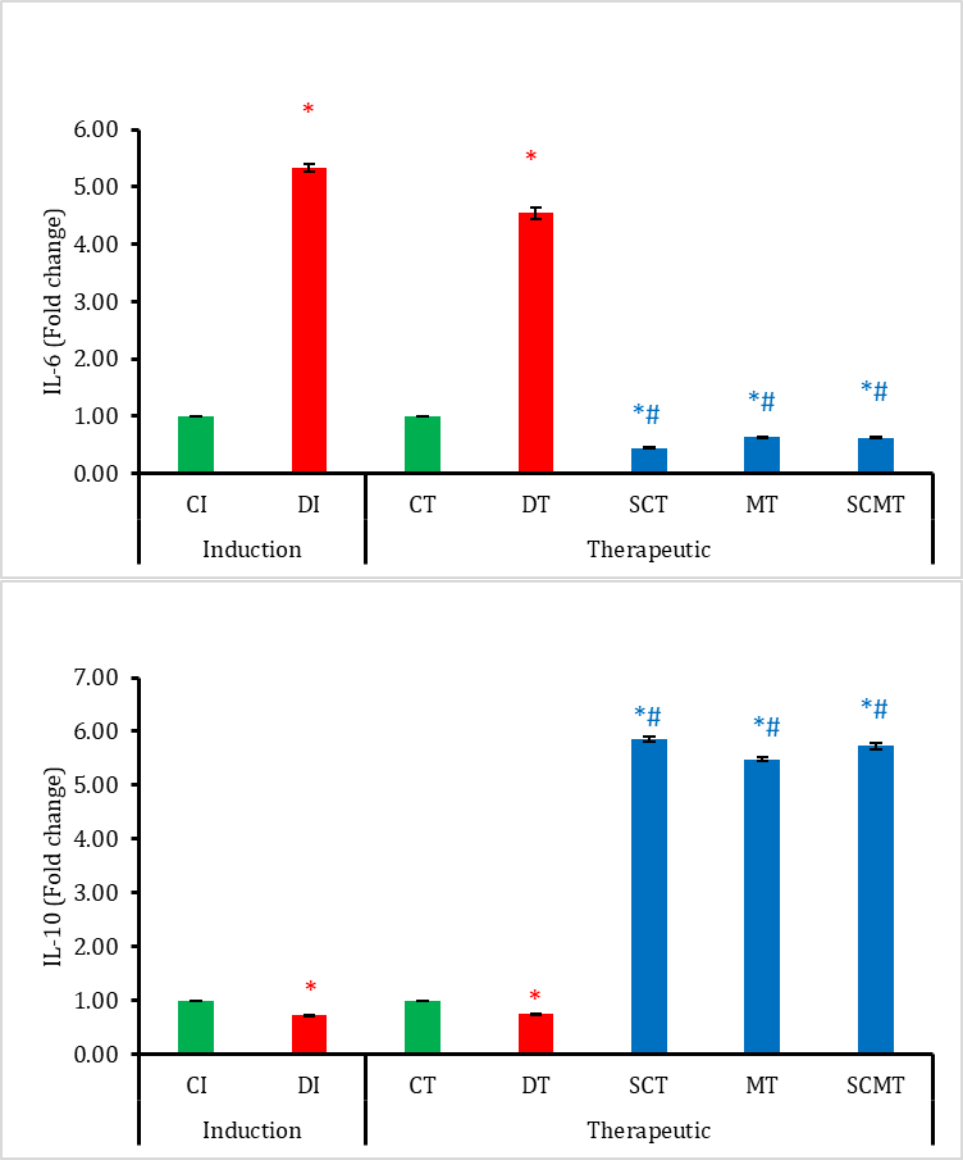


Fig 6. The gene expression levels of IL-6 and IL-10 in the kidney of all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

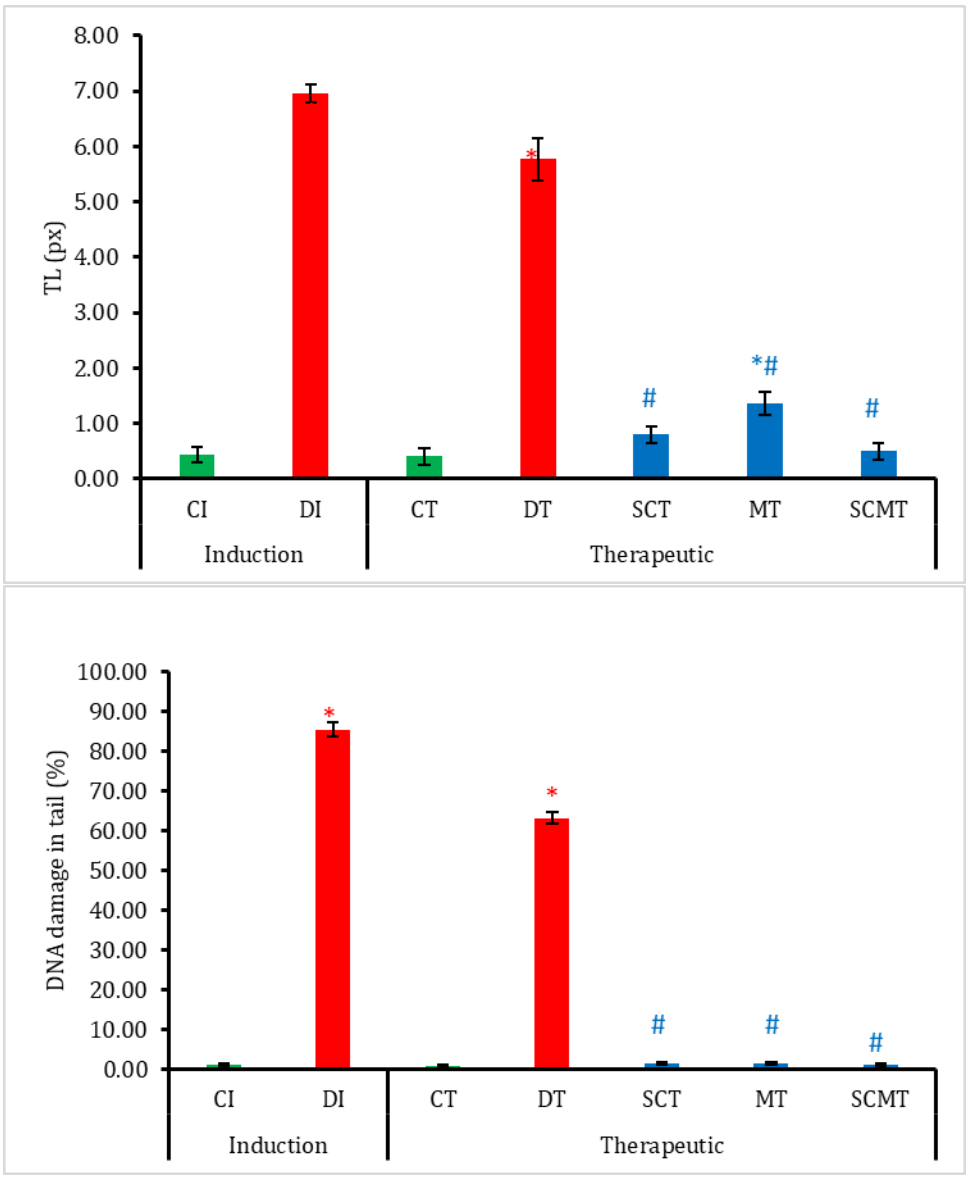


Fig 7. The Alkaline COMET parameters (TL & %DNA damage in tail) in the kidney of all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

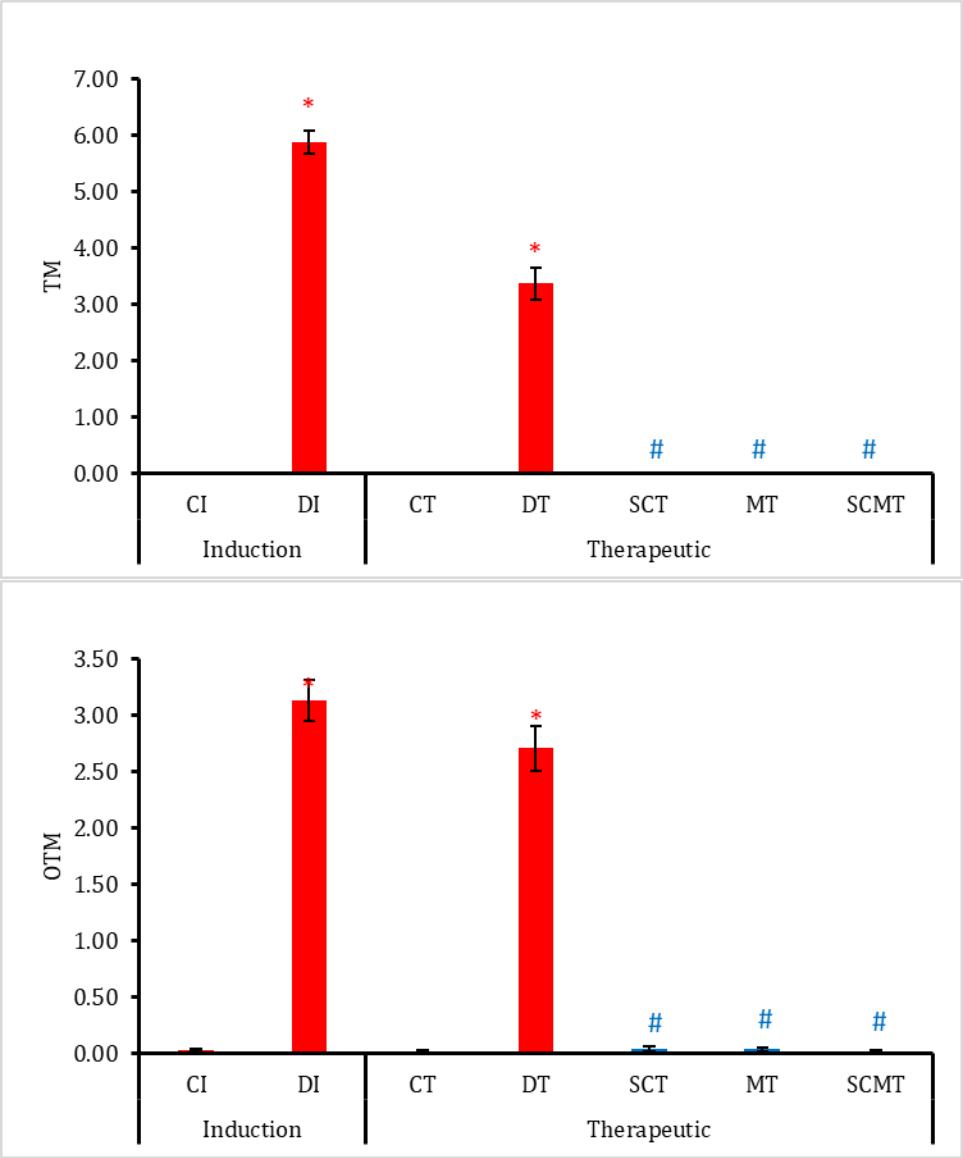


Fig 8. The Alkaline COMET parameters (TM & OTM) in the kidney of all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

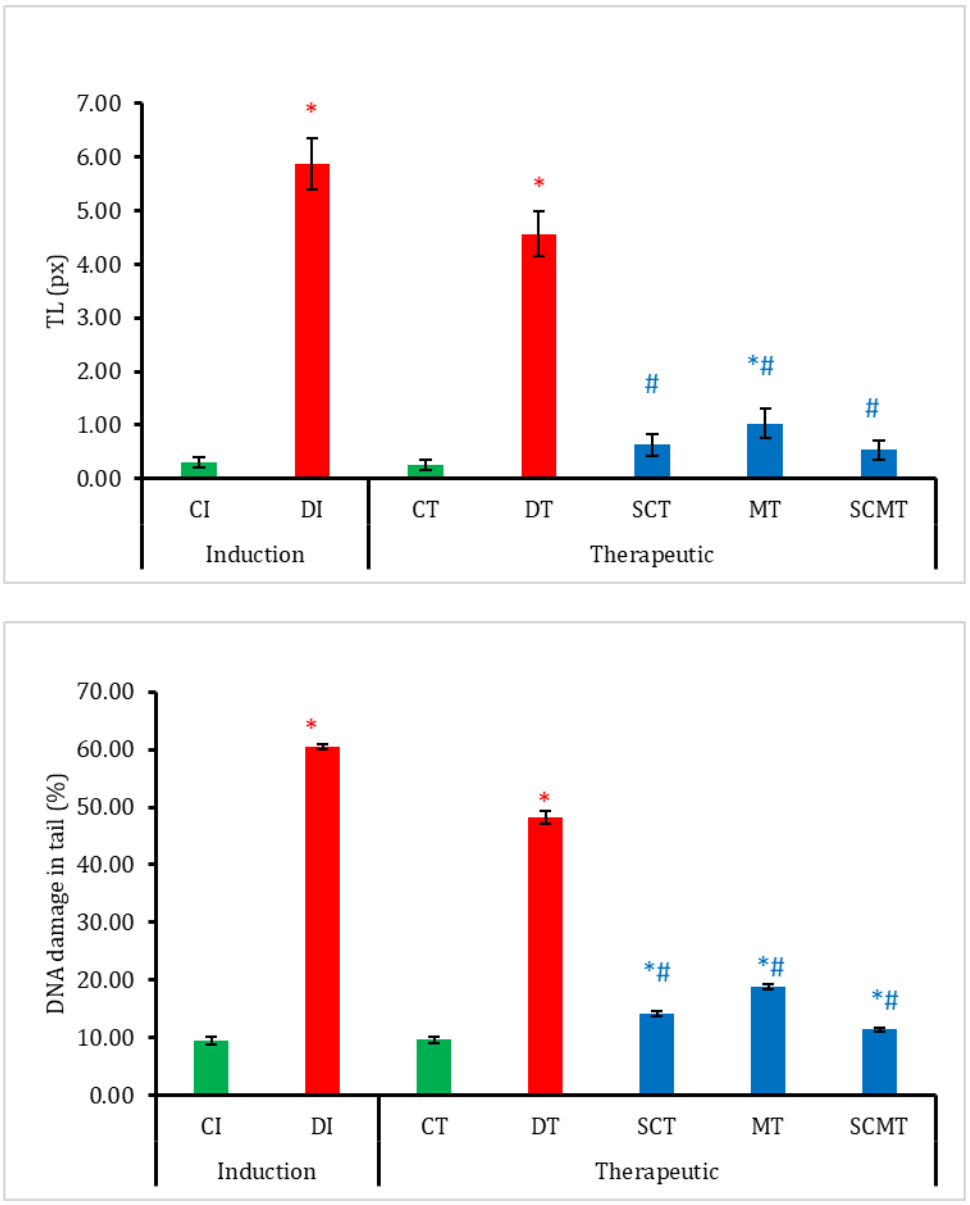


Fig 9. The Neutral COMET parameters (TL & %DNA damage in tail) in the kidney of all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

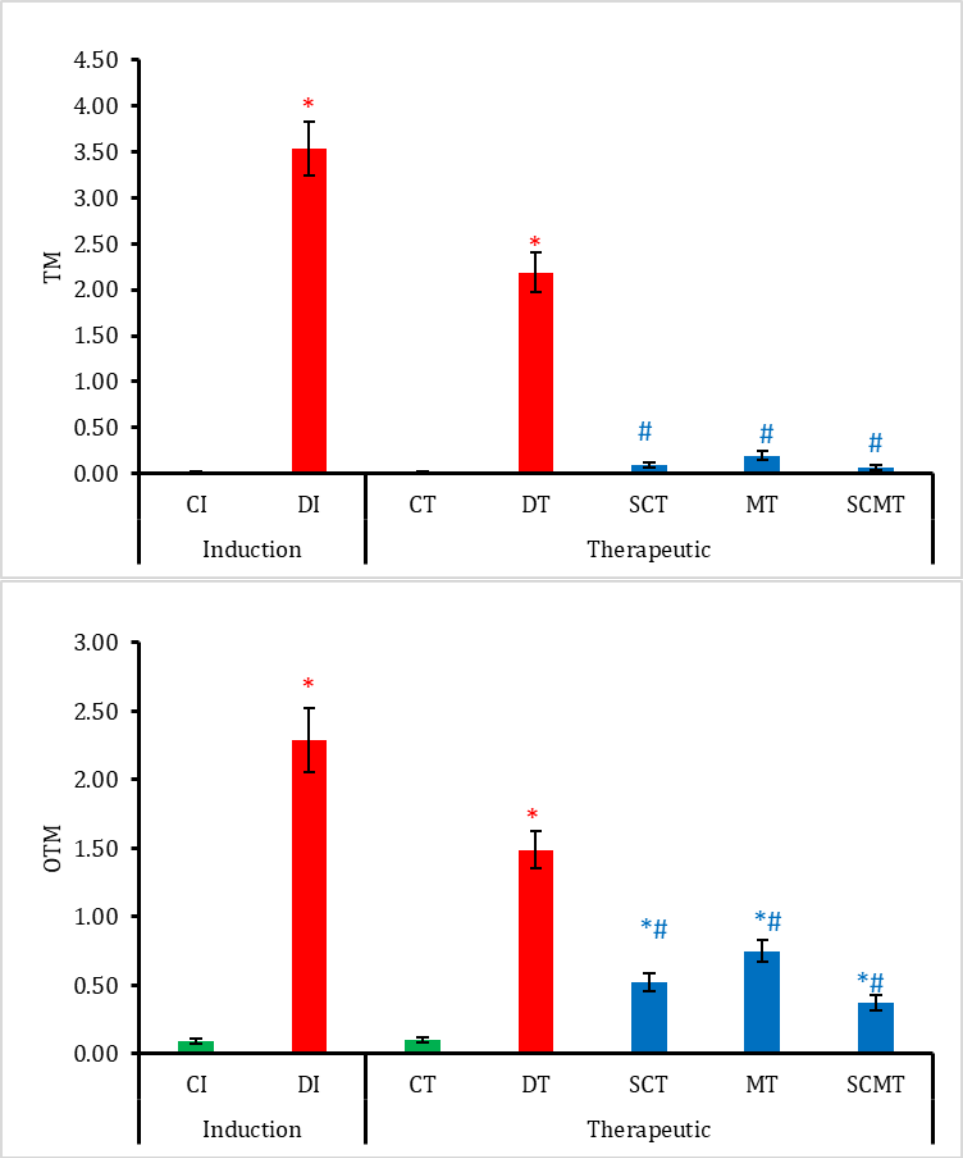


Fig 10. The Neutral COMET parameters (TM & OTM) in the kidney of all groups. Data are presented as mean ± standard error. *: significant difference at p1<0.000, as compared to the corresponding control group. #: significant difference at p2<0.000, as compared to the DT group.

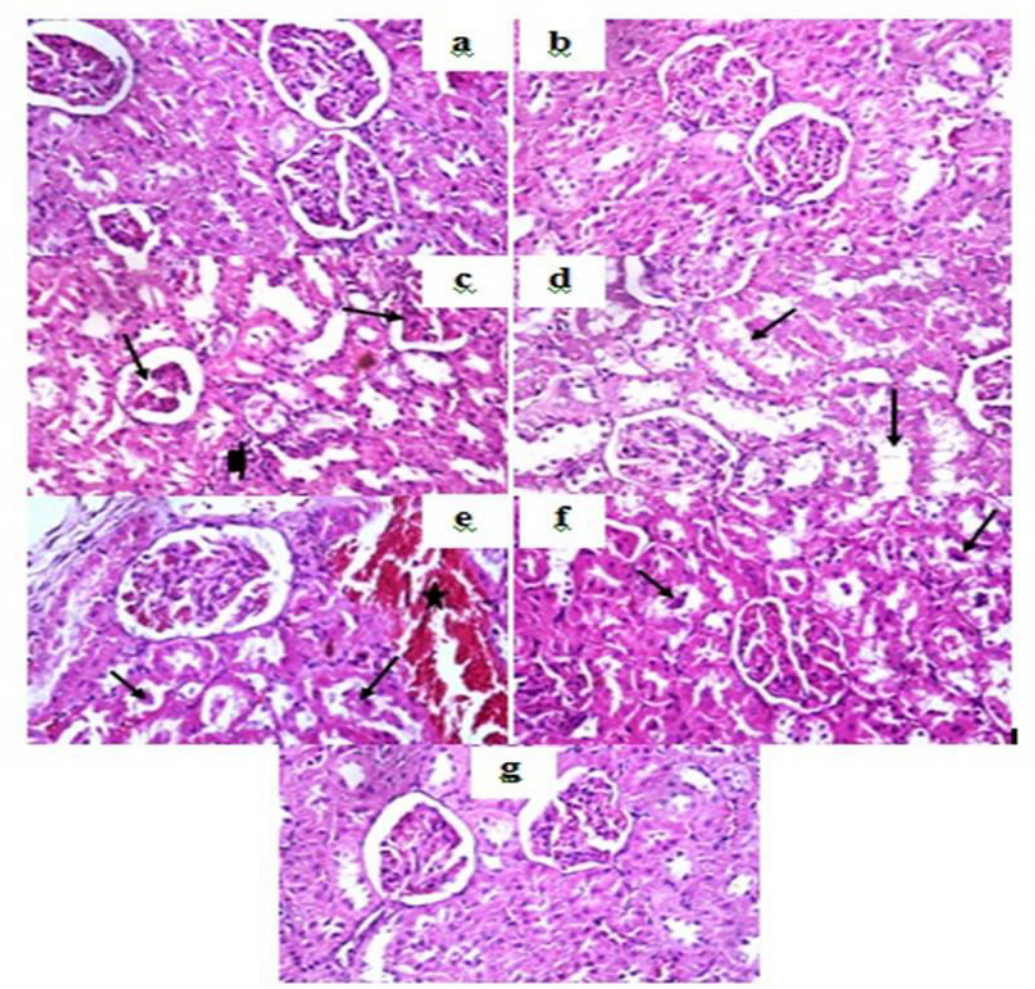


Fig. 11: Photomicrographs of Kidneys from different experimental groups stained with Hematoxylin & Eosin X400 showing; a & b- Induction & Therapeutic Control negative groups with normal renal parenchyma; note the normal glomeruli, normal renal tubules, and normal interstitial tissue. c- DOXO induction group with glomerular degeneration and atrophy. Interstitial tissue showing mononuclear cell infiltration. d- Doxo therapeutic group with diffuse renal tubule degeneration and necrosis. e- Stem cells group with congestion in the interstitial blood vessel (*) and cellular cast inside the renal tubules. f - Melatonin group with cellular cast inside the renal tubules, there are no signs of necrosis or congestion. g- Stem cells + Melatonin group with apparently normal renal parenchyma with neither signs of congestion nor fibrosis nor necrosis.

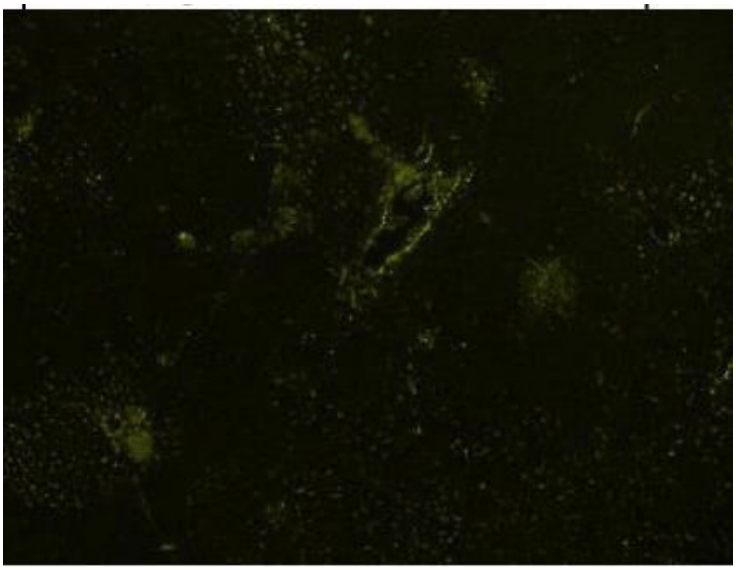


Fig. 12: Homing of Fluorescent ADMSCs in Rat hepatic Tissue. Image showing the migration of labeled ADMSCs to the liver two days post-injection in the rat model.

Discussion:

Cancer chemotherapy often leads to severe acute toxicity in various organs, particularly the kidneys. Alleviating these side effects while maintaining the effectiveness of chemotherapy against cancer is crucial. One proposed solution is combining drug delivery methods with potent antioxidants to lessen oxidative stress induced by chemotherapeutic drugs. This approach could potentially reduce organ damage without compromising therapeutic efficacy. The current study assessed the effects of Adipose-derived mesenchymal stem cells (ADMSCs) administration on renal injury induced by Doxorubicin (DOX). Our findings showed that melatonin and/or MSC significantly improved kidney tissue structure, biochemistry, and molecular function by reducing oxidative stress and suppressing hepatocyte apoptosis, counteracting the toxic effects of DOX.

This study highlights the impressive and promising effects of melatonin and ADMSC treatment in improving outcomes for DOX-induced kidney toxicity, warranting a detailed explanation of the underlying mechanism based on our findings.

In our experiment, DOX caused significant reductions in total body weight and kidney weight indicating severe renal toxicity, which was ameliorated by melatonin and/or ADMSC treatment. Previous studies, including those by Hassan et al., (2014), Samancı et al., (2022), and

Mansouri et al. (2017), have reported weight loss in DOX-treated groups due to muscles and adipose tissue loss from appetite suppression and metabolic alterations. Others, such as Najafi et al., (2020) observed that melatonin co-treatment reversed increased mortality and weight loss. Additionally, Abdelaziz et al., (2019) observed weight gain and improved renal weight with treatment. Our findings highlight that melatonin and ADMSCs, alone and together, reduce DOX-induced renal damage and weight loss, emphasizing their therapeutic potential. DOX accumulation in vital organs causes fibrosis, with toxicity depending on cumulative dose, leading to body weight loss and high mortality at 20–25 mg/kg, as evidenced by this study's findings of significant mortality and body and organ weight reduction (Wen et al., 2024).

Our study deduced that DOX significantly elevated oxidative stress biomarkers (MDA and NO) and reduced antioxidant biomarkers (CAT, SOD, and GSH) in kidney tissues, indicating severe renal toxicity, which was reversed with melatonin and/or ADMSCs. DOX- induced oxidative stress was attributed to its reactive intermediate and subsequent production of ROS, causing cellular damage (Shivakumar et al, 2012; Mansouri et al, 2017). Melatonin and antioxidants relieve DOX-induced oxidative stress by boosting antioxidant enzymes and reducing oxidative stress markers, and modulating gene expression enhancing its antioxidative, anti-inflammatory and anti-apoptotic properties (Afsar et al., 2020; Hajra et al., 2017b; Galano & Reiter, 2018). Combined with MSCs, melatonin ameliorates ROS accumulation and upregulates antioxidant genes, suggesting a synergistic therapeutic potential Samancı et al. (2022) Vohra et al. (2021b). Our study suggests that combining melatonin with ADMSCs synergistically reduces ROS accumulation and upregulates antioxidant genes, showing promise for treating DOX-induced renal damage and necessitating further safety assessments (Samancı et al., 2022; Vohra et al., 2021b).

DOX disrupts p53 target genes involved in the cell cycle, DNA damage response, apoptosis, mitochondrial function, ROS, and inflammation. It also affects DNA replication and generates ROS, leading to oxidative stress, mitochondrial dysfunction, and macromolecular damage to DNA, RNA, and proteins.

Oxidative DNA damage from oxidative stress, confirmed biochemically by elevated alkaline comet parameters, was observed in the kidney of DOX groups compared to controls.

(Kang et al., 2013; Shaltout et al., 2022; Kuchařová et al., 2019). These results indicate DOX-induced genotoxicity, which were reversed by melatonin and\or ADMSCs. Najafi et al., 2020 demonstrated that DOX increases NO, leading to the production of free radicals and reactive nitrogen species (RNS), thus increasing the oxidative stress and causing DNA damage (Rezvanfar et al., 2012; Najafi et al., 2020). El-Moneim et al. (2018) and Hajra et al. (2017b) reported increased DNA fragmentation and damage markers in DOX-treated rodents, consistent with our findings and those of Martins et al. (2012), Baumgartner (2004), L'Ecuyer et al. (2006), and Shokrzadeh et al. (2020) who observed similar genotoxic effects in various cell types. Afsar et al., (2020) showed that antioxidant treatment reverses significant DNA damage and restores DNA integrity in DOX-induced nephrotoxicity in rats, with melatonin reducing DNA damage through antioxidant effects, elevation of PARP enzyme activity, inhibition of DNA strand breaks, and restoration of mitochondrial membrane potential (Curtin and Szabo, 2013; Tarocco et al. 2019; Jajte et al., 2001; Najafi et al., 2020). Our comet assay results showed that melatonin and ADMSC treatments reduced DNA damage in DOX-induced renal toxicity, with their combined use suggesting a synergistic therapeutic approach for kidney health.

Histopathological examination revealed distinct renal lesions in our study, with normal renal architecture in control kidneys, glomerular degeneration and tubular necrosis in DOX-treated kidneys and remarkable improvement with ADMSCs and/or melatonin treatment, showing optimal renal health and synergistic therapeutic effects, aligning with earlier studies on DOX-induced renal alterations and the protective roles of melatonin and mesenchymal stem cells (Afsar et al., 2020; El-Moselhy and El-Sheikh, 2014) (Bilginoğlu, 2014; Najafi et al., 2020).

Our molecular measurements showed that DOX induction increased pro-inflammatory IL-6 and decreased anti-inflammatory IL-10, both of which were attenuated by melatonin and/or ADMSC treatment, consistent with findings by El-Moselhy& El-Sheikh (2014), who also observed elevated TNF-α and NF-κB expression levels in DOX-treated kidneys and liver. Our results indicated that DOX-induced oxidative stress creates a ROS-rich microenvironment, leading to endothelial cell injury, leukocyte infiltration, and increased pro-inflammatory cytokines such as TNF-α, COX-2, ICAM-1, and IL-6, hence inducing tissue inflammation (Pecoraro et al., 2016; Abdel-Daim et al., 2017; Aminjan et al., 2019; Delgado et al., 2004; Najafi et al., 2020). Melatonin, known for its antioxidant properties and inhibition of NF-κB

activation, reduces DOX-induced inflammation and pro-inflammatory cytokines (Arinno et al., 2021; Durdağı et al., 2021), and modulates immunomodulatory factors like IL-1β, TNF-α, and IL-6, enhancing the MSCs survival and therapeutic efficacy (Heo et al., 2019). Vohra et al. (2021).

Our findings are consistent with reports showing that DOX impairs kidney function, elevates proinflammatory cytokines, and reduces anti-inflammatory cytokines in rats (Tadros et al., 2024).

Assessing gene expression levels for apoptotic and inflammatory genes is crucial for evaluating the therapeutic effects of melatonin and ADMSCs against DOX-induced nephrotoxicity. This analysis provides insights into how melatonin and ADMSCs modulate the inflammatory cascade in kidney tissue, potentially attenuating nephrotoxicity and aiding in developing effective strategies for mitigating DOX-induced kidney damage. Here DOX significantly modulates gene expression by reducing the levels of anti-apoptotic Bcl2 and elevating pro-apoptotic gene p53, effects which were attenuated by melatonin and/or ADMSCs treatment. Anthracyclines like DOX induce tissue damage through ROS production, promoting oxidative stress and DNA damage, consequently altering the expression of cell cycle regulators such as P53 and Rb genes, leading to apoptosis or cellular senescence (Piegari et al., 2013). El-Moselhy and El-Sheikh (2014) observed that DOX increases the expression of the pro-apoptotic protein Bax in kidney and liver tissues. Mantawy et al. (2014) found that DOX enhances the expression of NF-κB, COX-2, and iNOS while suppressing Bcl2 expression, initiating the apoptotic cascade. This generates apoptotic cells that release damage-associated molecular patterns (DAMPs), activating pattern recognition receptors (PRRs), and exacerbating the immune response (Krysko et al., 2012). DOX also inhibits nuclear topoisomerase IIβ activity, leading to DNA breaks and transcriptional alterations (Zhang et al., 2012). Hajra et al. (2017b) demonstrated that DOX intoxication in mice resulted in significantly decreased levels of the anti-apoptotic marker Bcl-2 and increased levels of the pro-apoptotic marker Bax in cardiac tissue. Additionally, MSCs administration after DOX treatment has been demonstrated to reduce apoptosis and improve renal function (Gopinath et al., 2010; Di et al., 2012). Melatonin has been shown to enhance Bcl2 protein levels and reduce pro-apoptotic proteins like Bax and caspase 3 in DOX-induced apoptosis (Liu et al., 2018). Additionally, melatonin modifies DOX-induced

elevations in caspases 3 and 7 activity and PARP cleavage, suggesting a role in apoptosis regulation (Chua et al., 2016; Liu et al., 2008; Sishi et al., 2013; Najafi et al., 2020).

The Neutral Comet Assay is commonly used to detect double-strand DNA Breaks and identify potential apoptotic windows (Lu et al., 2017; Roy et al., 2021; Husseini et al., 2005). Therefore, we utilized this assay in our study to confirm the molecular results of apoptosis. Hajra et al. (2017b) indicated that DOX-treated mice exhibited significantly elevated chromosomal aberrations, such as stretching, acentric fragment, GAP, sister-chromatid union, and constriction in metaphase plates. Our comet assay analysis revealed increased DNA damage in various tissues, evidenced by higher percentages of damaged cells, tail DNA (%), average tail length, and Olive tail moment. Additionally, DOX administration led to significant DNA fragmentation, characterized by oligonucleosome length degradation and mixed laddering and smearing of DNA fragments observed through agarose gel electrophoresis or DNA fragmentation assay. DOX treatment significantly increased apoptotic DNA damage, as indicated by elevated neutral comet assay parameters and apoptotic index in mice compared to the vehicle control group (Hajra et al., 2017b).

Melatonin has shown protective effects against the genotoxicity induced by cyclophosphamide, a chemotherapeutic drug and immunosuppressant (Shokrzadeh et al., 2014). Skubis-Sikora et al., 2023 used an Annexin/IP assay to assess the effects of melatonin on ADMSC apoptosis and necrosis, finding that the percentage of live cells was significantly higher compared to the apoptotic or necrotic cells in all investigated cultures. The presence of early-apoptotic, late-apoptotic cells and very few necrotic cells indicates that melatonin does not induce apoptosis in ADSCs according to Hajra et al., (2017b). The significant elevation in the neutral comet assay parameters detecting the apoptotic DNA damage aligns with findings that the apoptotic index (%) increased significantly in mice treated with DOX compared to the vehicle control group due to DOX-induced toxicity (Hajra et al., 2017b).

Conclusion:

We confirm that melatonin and\or ADMSCs, possess antioxidant, anti-nitrosative, anti-inflammatory and anti-apoptotic properties, which can alleviate DOX-induced hepato-renal toxicity by disrupting the toxic vicious circuit induced by DOX. Our results suggest melatonin

and\or ADMSCs as effective successful adjuvant therapies for DOX chemotherapy, potentially broadening the therapeutic window of DOX as an anticancer drug. Overall, our findings support the combined use of melatonin and ADMSCs in treating DOX-induced renal damage, highlighting their synergistic potential in reducing oxidative stress and inflammation. Further studies are warranted to explore their clinical applications and safety profiles.

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Statements and Declarations

Competing Interests:

The authors have no relevant financial or non-financial interests to disclose.

Data Availability:

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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