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Research article

Melatonin mitigates doxorubicin induced chemo brain in a rat model in a NRF2/p53–SIRT1 dependent pathway

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ABSTRACT

Cancer is a critical health problem, and chemotherapy administration is mandatory for its eradication. However, chemotherapy like doxorubicin (Dox) has serious side-effects including cognitive impairment or chemo brain. Melatonin is a neuroprotective agent that has antioxidant, and anti-inflammatory effects. We aimed to explore melatonin's effect on Dox-induced chemo brain to discover new mechanisms associated with Dox-induced neurotoxicity and try to prevent its occurrence. Thirty-two male albino rats had been equally divided into four groups; control, melatonin-administrated, Dox-induced chemo brain, and melatonin + Dox treated. On the 9th day, brain had been excised after scarification and had been assessed for reactive oxygen species measurement, histopathological analysis, immunohistochemical, gene and protein expressions for the nuclear factor erythroid 2-related factor 2 (Nrf2), p53 and Silent information regulator 2 homolog 1 (SIRT1). Our results show that melatonin coadministration diminished Dox induced hippocampal and prefrontal cortex (PFC) cellular degeneration. It alleviated Nitric Oxide (NO) level and reversed the decline of antioxidant enzyme activities. It also upregulated Nrf2, SIRT1 and downregulated p53 gene expression in rats receiving Dox. Moreover, melatonin elevated the protein expression level of Nrf2, SIRT1 and reduced p53 corresponding to immunohistochemical results. The data suggested that melatonin can mitigate Dox-induced neurotoxicity by aggravating the endogenous antioxidants and inducing neurogenesis through activation of Nrf2/

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p53–SIRT1signaling pathway in adult rats' PFC. These effects were associated with Nrf2, SIRT1 activation and p53 inhibition. This could be guidance to add melatonin as an adjuvant supplement to Dox regimens to limit its adverse effect on the brain function.

1. Introduction

Combination of chemotherapy, especially in treating breast cancer, impaired the cognitive function [1–3]. Chemotherapy has some side effects including, memory impairment, loss of concentration, and language difficulty, which is called chemotherapy induced cognitive impairment or chemo brain [4,5]. This greatly affects the patients' quality of life, especially in cancer breast patients, who demonstrated the most impaired cognitive function following chemotherapy [6,7]. However, the exact mechanisms behind the cognitive impairment are unknown, various hypothesis have been postulated, like reduction in estrogen hormonal level, DNA damage or upregulation of cytokines [8].

Doxorubicin (Dox) is an antibiotic made by the fungus Streptomyces Peucetius; it is a powerful anticancer medication used frequently in treating cancers especially breast cancer [9]. However, it has dangerous effects on normal tissues [10,11] including cardiomyopathy [11] and brain cognitive impairment [12,13]. Dox-treated mice demonstrated a significant elevation in levels of lipid peroxidation and protein oxidation in their brains [14]. Dox also induced circulating tumor necrosis factor alpha (TNFα), activated glial cells in the cortex and hippocampus which was significantly reduced after blocking the circulating TNFα with anti-TNF antibody. Systemic Dox treatment also increased oxidative stress and caspase-3 apoptotic activity in the brain. The levels of p53 pro-apoptotic proteins, Bax and Bcl-xL anti-apoptotic protein were also elevated in the brain after Dox administration [15].

Melatonin (N-Acetyl-5-methoxytryptamine) is a hormone built in the pineal gland from L-tryptophan, it's also generated in the peripheral tissues like the retina, gastrointestinal tract, skin, and the immune system [16,17]. It is well-known of its role in regulating circadian rhythm, it also has anti-inflammatory, antioxidant, anti-apoptotic, immunomodulatory, and neuroprotective effects [16, 18–20].

Reactive oxygen species (ROS) are highly reactive unstable molecules, its excessive production or failure of antioxidant defense mechanisms causes oxidative stress, cellular damage and ends with several diseases [21]. The transcription factor nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) enhances antioxidant transcription, anti-inflammatory and cytoprotective genes [22]. In oxidative stress, Nrf2 is translocated to the nucleus [22]. Various studies have suggested that melatonin anti-inflammatory and antioxidant effect is through activation of Nrf2 in microglial cells [23,24]. Melatonin also was demonstrated to increase brain expression of Sirtuin 1 (SIRT1), which is a part of the silent information regulator 2 protein family. Its neuroprotective effect is through activation of SIRT1/Nrf2 signaling pathways [25]. SIRT1 regulates many processes like aging, cell survival, homeostasis, metabolism, and inflammation [26]. SIRT1 deacetylates many target genes like p53, nuclear factor kappa (NF-κB), and controls its activities. It is broadly expressed in the brain and maintained its physiological functions via its neuroprotective and anti-inflammatory effects [27, 28]. The p53 marker is an important substrate of SIRT1, its acetylation is critical for its function and stabilization, p53 can attach to two SIRT1 exponent regions directly and repress SIRT1 transcriptional activity. Nrf2 increased the expression of SIRT1 by decreasing p53 protein levels [29–31]. Melatonin demonstrated antioxidant effects against Dox induced oxidative damage in many tissues including the brain, kidney, heart, liver, and lungs [32–34]. However, the exact mechanism behind Dox induced chemo brain and the protective role of melatonin is still unknown.

Thus, the current study was designed to assess whether Dox-induced neurotoxicity can be averted by melatonin supplementation. The role of Nrf2/p53–SIRT1 signaling pathway in the pathogenesis of Dox-induced neural injury and melatonin neuroprotective role was also studied using various cellular and molecular mechanisms.

2. Methods and matertials

This experimental study was carried out at the Veterinary Medicine Faculty, University of Mansoura, Egypt.

2.1. Experimental design

Thirty-two male adult albino rats, each weighing 150–250 gm, were divided equally into 4 groups; **negative control** was given an intraperitoneal injection (i.p.) of saline for 7 days, then the rats were sacrificed after 2 days to be compared with the positive control group, and with the treated group. **Melatonin-administrated group** was given melatonin dissolved in saline (40 mg/kg/day, i.p.) for 7 days [33,35]. **Positive control group,** a cumulative dose of Dox was injected (18 mg/kg, i.p.) on the 5th, 6th and 7th days to induce neural toxicity [33,35], then the rats were sacrificed after 2 days to confirm the development of chemo brain. **Treated group (melatonin + DOX)** was given melatonin (40 mg/kg/day, i.p.) for 7 days and Dox (18 mg/kg, i.p.) on the 5th, 6th and 7th days. After 2 days of following Dox injection, the rats were sacrificed.

2.2. Morris water maze test

The Morris water maze test was used for behavioral evaluation of learning capacity and spatial memory, it is formed of learning and retention stages. A large circular pool is used for this test. It's measured 150 cm in diameter, height of 45 cm, and 30 cm of water was

filled to a depth at 28 °C, it was divided equally into 4 quadrants. Then in one quadrant of the pool, a circular platform (4.5 cm diameter) was placed for learning memory. Each rat was exposed to 4 succeeding trials every 5 min. Each rat was then given 120 s to reach the platform. If the animal failed to locate the platform within 120 s, it was directed to it. The time needed by each rat to locate the platform was measured. Then 4 h following the last learning session, the platform was removed, and the time required to reach the platform quadrant was recorded as memory retention [36].

2.3. Specimen collection

On the 9th day, rats were euthanized by the administration of sodium thiopental (1 ml, i.p) and the prefrontal cortex (PFC) and hippocampus was dissected. Parts of the PFC and hippocampus were fixed in 10 % formalin and preserved for histological and immunohistochemical examination. Another PFC part was homogenized for the oxidative stress markers measurement. A 3rd PFC portion was freshly retained in RNA later for real time PCR and western blotting.

2.4. Oxidative stress markers assessment

The PFC and hippocampal tissues were homogenized in 5 mmol/l Tris-HCl (containing 2 mmol/l EDTA, 7.4 pH), then centrifuged at 1000×*g* for 10 min at 40 °C. Homogenate supernatants were utilized for Nitric Oxide (NO), and reduced glutathione (GSH) activity measurement, after wet weight per ml calculation [37]. NO level in the homogenates was measured using Griess reagents by colorimetric detector of nitrite levels [38], utilizing the nitric oxide assay kits (2533; Bio-diagnostic Com.). For colorimetric detection of GSH, assay kits (TA2511; Bio-diagnostics) were used. Based on colorimetric principle, the absorbance was measured spectrophotometrically at specific wave lengths following the manufacturer's instructions.

2.5. Histological, and immunohistochemical studies

Brain hippocampal and PFC specimens were cut, fixed, embedded in paraffin, and prepared for hematoxylin and eosin (H&E) stain. Immunodetection was performed on Nrf2, p53 and SIRT1 markers in the PFC The tissues were fixed using 4 % paraformaldehyde, stabilized in 0.2 % Triton X-100, nonspecific receptors were blocked for 30 min with normal goat serum mixed in phosphate buffered saline (PBS), then were incubated 12 h at 4 °C with the primary antibodies; p53 rabbit monoclonal (ab227655; 1:100 dilution, Abcam), Nrf2 rabbit polyclonal (ab31163; 1:100 dilution, Abcam) and SIRT1 rabbit monoclonal (ab189494; 1:500 dilution, Abcam). Samples were washed with 0.1 % PBS/bovine serum albumin (BSA), then incubated for 2 h at room temperature with the correlated secondary antibodies and the recommended dilution (1:50), Diaminobenzidine (DAB) and Streptavidin peroxidase were utilized to gain the brown color, Mayer's hematoxylin was used to counterstain for 30 s, then washed three times with PBS and once with distilled water [39]. Finally, slides were mounted, and an Olympus microscope (CX41) was used for examination.

2.6. Morphometric studies

Five randomized non-overlapped sections' fields from each rat were examined. The area percentage of the brown immune stained Nrf2, p53 and SIRT1 sections was measured in each group (× 200), using the NIH (National Institute of Health) Image J program. The brown-shaded immunohistochemically stained pixels were separated from the background using the Image J program. The saturation and the brightness for the selected images were adjusted to define color threshold scale for the brown-stained immunohistochemically stained areas. Finally, the chosen threshold was applied to segregate the positively stained color pixels (DAB) from the background (H&E-stained pixels) from in the selected images [40].

2.7. Quantitative real-time PCR

Samples of the rats' PFC (50 mg each) were preserved in RNA later (500 μl) till processing for mRNA quantification of the Nrf2, p53 and SIRT1 genes by quantitative real-time reverse transcription-PCR (qRT-PCR). Liquid nitrogen was used to homogenize the tissue specimens and QIAzol reagent (Qiagen, Germany) was utilized for total RNA extraction. Thermo Scientific NanoDrop One (USA) examined the RNA yield to determine its concentration and purity. Using a Proflex Thermal Cycler (Applied Biosystems, USA) and SensiFASTTM cDNA synthesis Kit (Bioline, UK), first strand cDNA was harmonized from 1 μg of RNA. Primers were annealed for 10 min at 25 °C, reverse transcription was executed for 15 min at 42 °C, and inactivation took place at 85 °C for 5 min.

Amplification of cDNA templates was conducted using Azure Cielo 6 (Azure, USA) real-time PCR equipment, 10 μL of PCR green Bioline SYBR Master Mix (Bioline, UK), 1 μL of cDNA template, 2 μL (10 pmol/μl) of gene primer, and 7 μL of free water nuclease made up the 20 μl reaction volume. After adjusting the thermal profile for 2 min at 95 °C, there were 40 cycles: 5 s of denaturation at 95 °C, then 30 s of annealing at 60 °C (62 °C with p53) [41].

The rat primers' sequences allocated for qRT-PCR examination were taken from previous publications as demonstrated in Supplementary Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as a normalized gene. All primers' specificity was inspected through the Primer BLAST program [https://www.ncbi.nlm.nih.gov/tools/primer-blast/]. A melting curve inspection was performed to verify the PCR products' specificity. Primer sets were acquired from Vivantis (Vivantis Technologies, Malaysia), levels of gene expression were displayed as ΔCt = Ct target gene– Ct housekeeping gene; $2^{-\Delta\Delta CT}$ procedure was utilized to measure gene expression fold change as mentioned [42]. PCR were run on a 3% agarose gels and photographs acquired through Azure 600

documentation gel system (Azure, USA).

2.8. Western blotting analysis

Homogenized samples from the lesion sites of the brain were used for immunoblotting to assess Nrf2, p53 and SIRT1 protein levels. The rats' prefrontal cortical homogenates were prepared at pH 7.4 in cold lysis buffer containing Tris-Base (10 mM), EGTA (25 mM), EDTA (25 mM), NP-40 (1 %), NaCl (50 mM), Triton X-100 (1 %), and immediately complemented with protease and phosphatase blockers (1:500, Sigma, St. Louis, USA). Procedure was performed as previously reported [43,44]. Quantification of proteins in the specimens was carried out using Pierce™ 660 nm colorimetric assay kit (Thermo Scientific, Rockford, USA), 30 μg proteins from each sample were boiled for 5 min in the loading buffer containing sodium dodecyl sulfate at 96 °C.

Then, an electrophoresis unit was used to separate the samples (Cleaver Scientific Ltd, UK), transferred on PVDF membranes using an Electro-blotter (Bio-Rad, Hercules, CA, USA), blocked in bovine serum albumin (BSA: 5 % in Tris-buffered saline with Tween-20), then incubated with rabbit polyclonal anti-SIRT1 (07–131; 1:100 dilution; Millipore, Burlington, MA, USA), rabbit monoclonal anti-

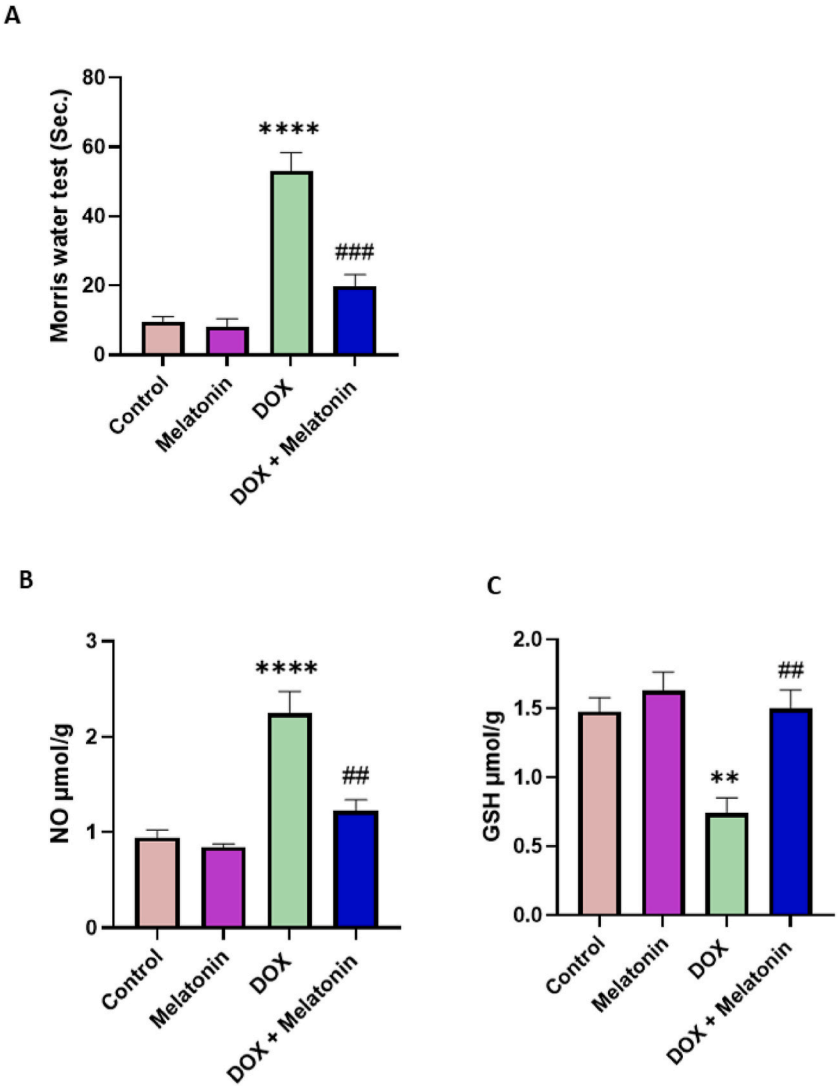


Fig. 1. Histograms demonstrating Morris water test results and the mean hippocampal and PFC tissue oxidative stress markers (NO and GSH). **(A):** Moris water test revealed a statistical elevation in spatial memory deficiency in the Dox group as compared to the control ($P < 0.0001$), which was reduced after the cotreatment with melatonin ($P < 0.001$). **(B):** Level of NO in different groups was demonstrated. The Dox-treated group showed a statistically significant upregulation in the level of NO compared to the control group, ($P < 0.0001$). However, it was downregulated in the DOX-melatonin cotreated group ($P < 0.01$). **(C):** The Dox group demonstrated a significant downregulation in GSH levels compared to the control group, ($P < 0.01$). Whereas it was upregulated significantly after cotreatment with melatonin ($P < 0.01$). One-way ANOVA was utilized to compare all the groups, and an unpaired *t*-test was applied to compare each group.

p53 (ab109396; 1:1500 dilution; Abcam, Cambridge, UK), rabbit polyclonal anti-Nrf2 (E-AB-32280; 1:500 dilution; Elabscience Biotechnology Inc., Texas, USA), and β-actin (A5060; 1:2000 dilution; Sigma-Aldrich, St. Louis, USA). After that, the membranes were washed with PBS and incubated with secondary antibody at room temperature for 1 h, the band signals were gained with Chemi-Doc imager (Bio-Rad, CA, USA). Finally, the protein bands' intensities were analysed with their corresponding β-actin bands using the ImageLab Software (Biorad).

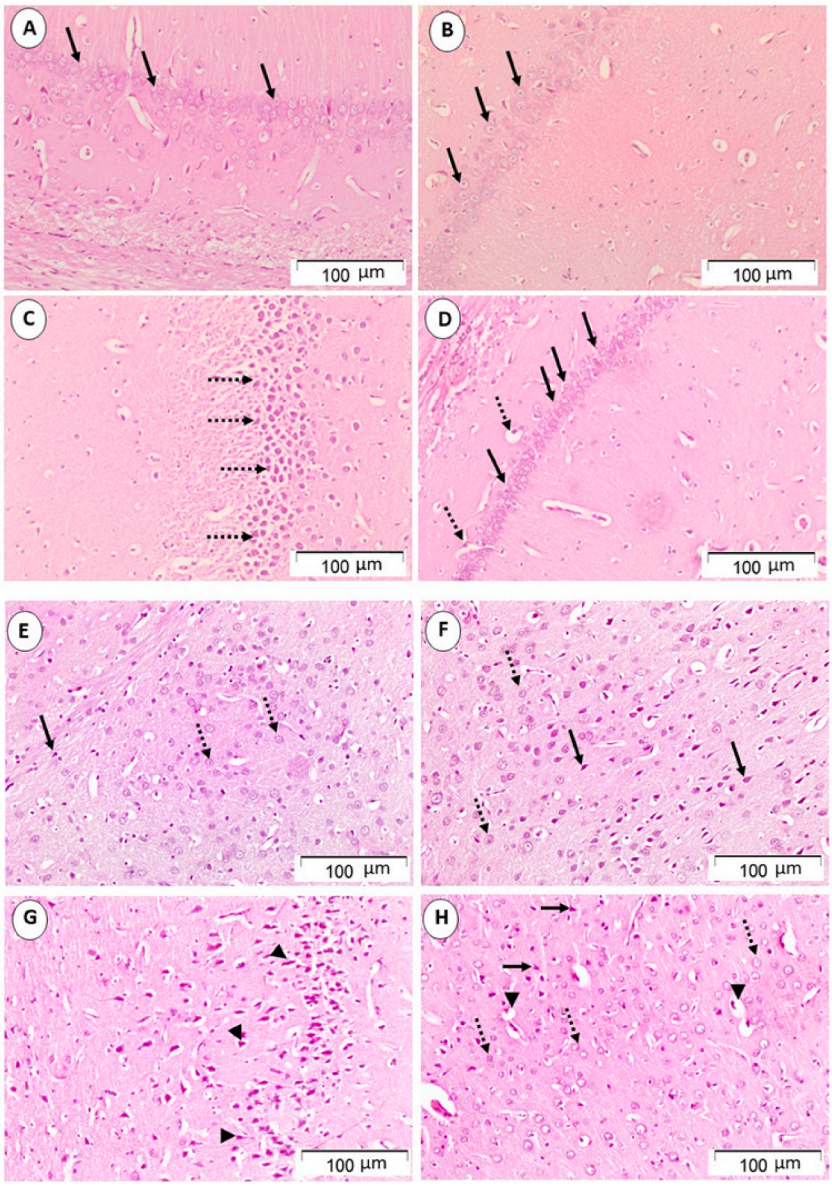


Fig. 2. Photomicrograph of hippocampal and PFC H & E-stained sections.
(A), (B): Control and melatonin-treated groups individually illustrated CA1 pyramidal cell layer that was composed of small pyramidal neurons with vesicular nuclei and prominent nucleoli (arrows). **(C):** The DOX-treated group displayed the pyramidal neurons mostly deeply stained with pyknotic nuclei (dotted arrows). **(D):** Combined melatonin and doxorubicin-treated group demonstrated pyramidal neurons predominantly with normal large vesicular nuclei and prominent nucleoli (arrows). A few deeply stained neurons surrounded by halos are still localized (dotted arrows).
(E), (F): Control and melatonin-treated groups with normal-appearing granule cells having rounded vesicular nuclei with prominent nucleoli (dotted arrows) and pyramidal cells with long peripheral processes and vesicular nuclei (arrows). (**G**) The DOX-treated group showed many deeply shaded neurons with pyknotic nuclei surrounded by halos (arrowheads). **(H):** Combined melatonin and doxorubicin-treated group displayed restoration of PFC architecture. Granule cells (dotted arrows) and pyramidal cells (arrows) seem normal. However, some deeply stained neurons and pyknotic nuclei adjoined by halos were still localized (arrowheads). **(H & E x 200)**.

2.9. Statistical analysis

The statistical analysis was operated with GraphPad Prism 10 (GraphPad Software Inc, CA, USA). One-way ANOVA was allocated for more than two groups' comparisons and unpaired *t*-test in the comparison between two groups. The numerical data were displayed as mean ± standard deviation and $P \leq 0.05$ was used as statistically significant.

3. Results

3.1. Behavioral assessment by Morris water maze test

The cognitive abnormalities were analysed through examining the behavioral performance. Dox treated rats showed memory deficits, as shown in the Morris water maze test. There was a significant increase in the time required to find the hidden platform in the

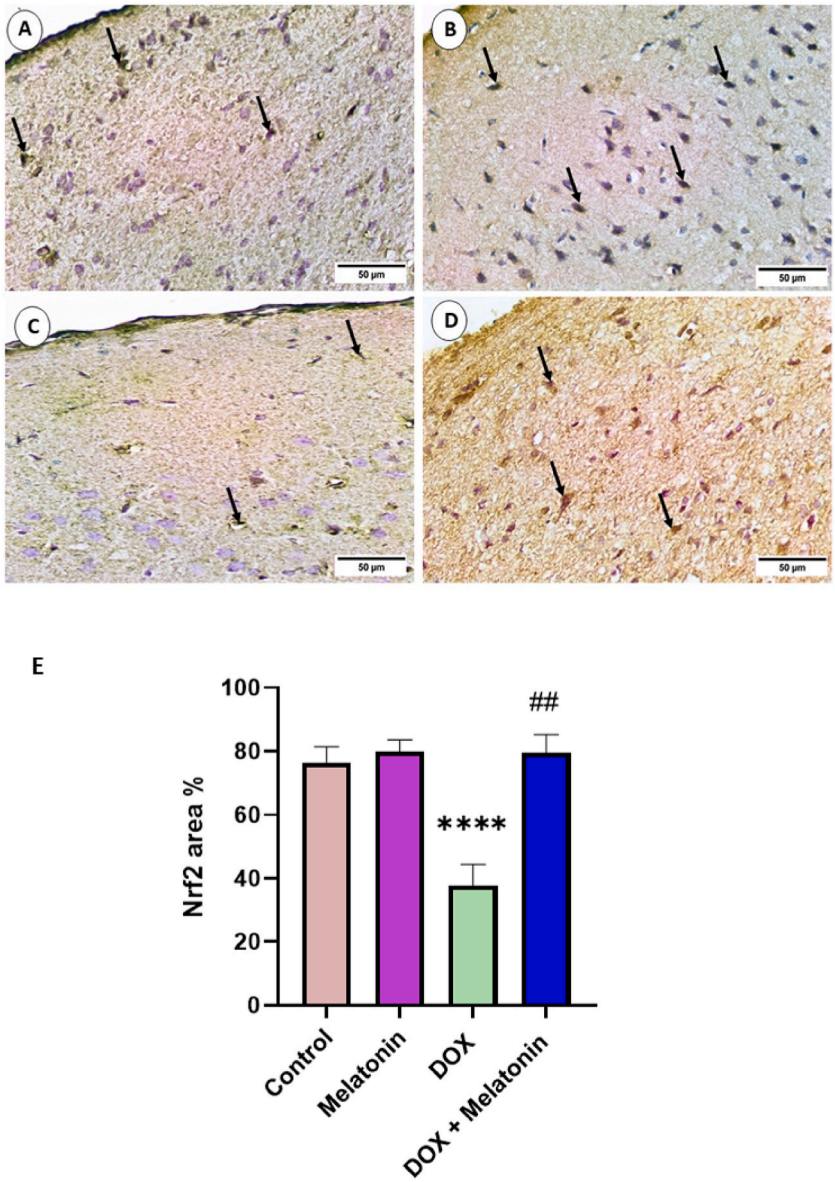


Fig. 3. Photomicrograph of Nrf2 immunostaining in the PFC sections.
(A), (B): Control and melatonin-treated groups showed strong positive Nrf2 reaction (arrows). **(C)** The DOX-treated group showed a weak positive Nrf2 reaction. **(D):** Combined melatonin and doxorubicin-treated group revealed moderate positive Nrf2 reaction (Nrf2 x 400). **(E):** Area percentage of Nrf2 demonstrated a statistical reduction in the DOX-treated group as compared to the control ($P < 0.0001$). and statistical elevation in the DOX + melatonin cotreated group as compared to the control ($P < 0.01$).

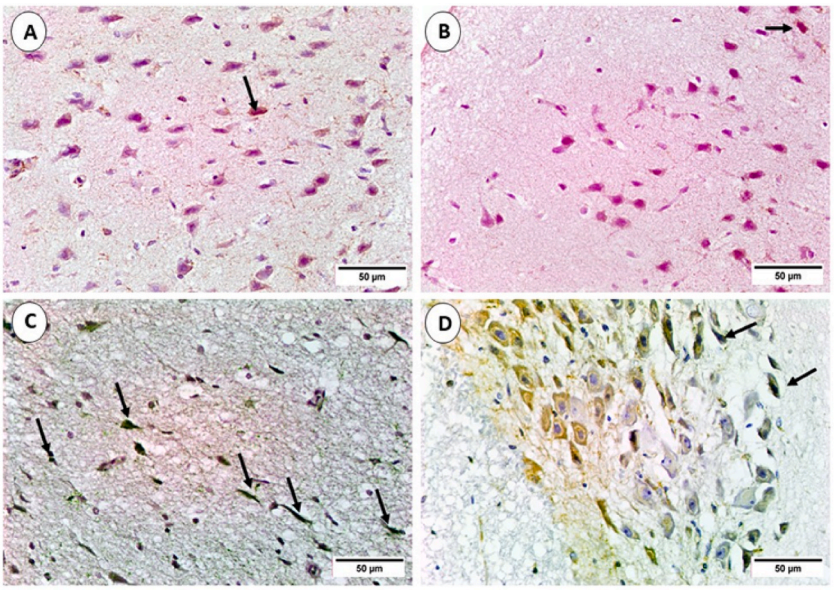
Dox treated group compared to the control ($P < 0.0001$), indicating spatial memory deficiency. However, melatonin co-treated rats showed a significant reduction in the latency time as compared to the Dox treated group ($P < 0.001$) (Fig. 1A).

3.2. Biochemical assessment of oxidative stress markers in the hippocampal and PFC homogenates

Lipid peroxidase investigation showed significant elevation in the NO concentration ($P < 0.0001$) and statistically significant lowering in the concentration of GSH ($P < 0.01$) after treatment with Dox compared to the control group. However, after the treatment with melatonin, NO concentration was reduced significantly as compared to the Dox treated group ($P < 0.01$), and GSH concentration increased also significantly as compared to Dox group ($P < 0.01$) (Fig. 1B and C).

3.3. Histopathological assessment of the hippocampal and PFC sections

H&E-stained sections of rats' hippocampus in the control group displayed Cornu Ammonis 1 (CA1) pyramidal cell layer with small



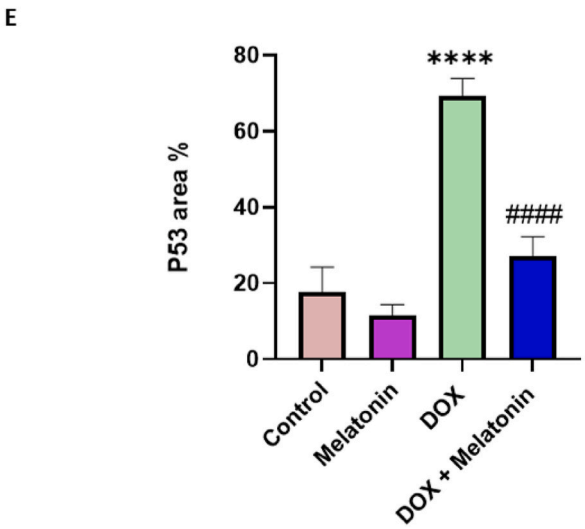


Fig. 4. Photomicrograph of p53 immunostaining in the PFC sections.
(A), (B): Control and melatonin-treated groups revealed weak p53 positive reaction (arrows). **(C)** The DOX-treated group showed a strong p53 positive reaction. **(D):** Combined melatonin and doxorubicin group displayed moderate positive P53 reaction (p53 x 400). **(E):** Area percentage of p53 demonstrated a significant elevation in the DOX-treated group compared to the control ($P < 0.0001$).

pyramidal neurons and enlarged vesicular nuclei and prominent nucleoli (Fig. 2A). The melatonin group displayed a similar architecture like the control section (Fig. 2B). However, the Dox-treated group showed deeply tinged pyramidal neurons with pyknotic nuclei (Fig. 2C). Treatment with melatonin demonstrated that pyramidal neurons came out predominantly normal with vesicular nuclei and prominent nucleoli, while some deeply stained neurons surrounded by halos were also noticed (Fig. 2D).

H&E-stained sections of the rats' prefrontal cortex in the control group, displayed the control and melatonin-treated groups with normal appearing granule cells, having rounded vesicular nuclei, prominent nucleoli (Fig. 2E) and pyramidal cells with long peripheral process and vesicular nuclei (Fig. 2F). However, the Dox-treated group showed numerous deeply shaded neurons with pyknotic nuclei surrounded by halos and many irregular elongated nuclei of typical microglial cells (Fig. 2G). Moreover, the combination of melatonin with Dox demonstrated restoration of PFC architecture. Granule and pyramidal cells seemed normal. However, some neurons stained deeply with pyknotic nuclei surrounded by halos were still demonstrated (Fig. 2H).

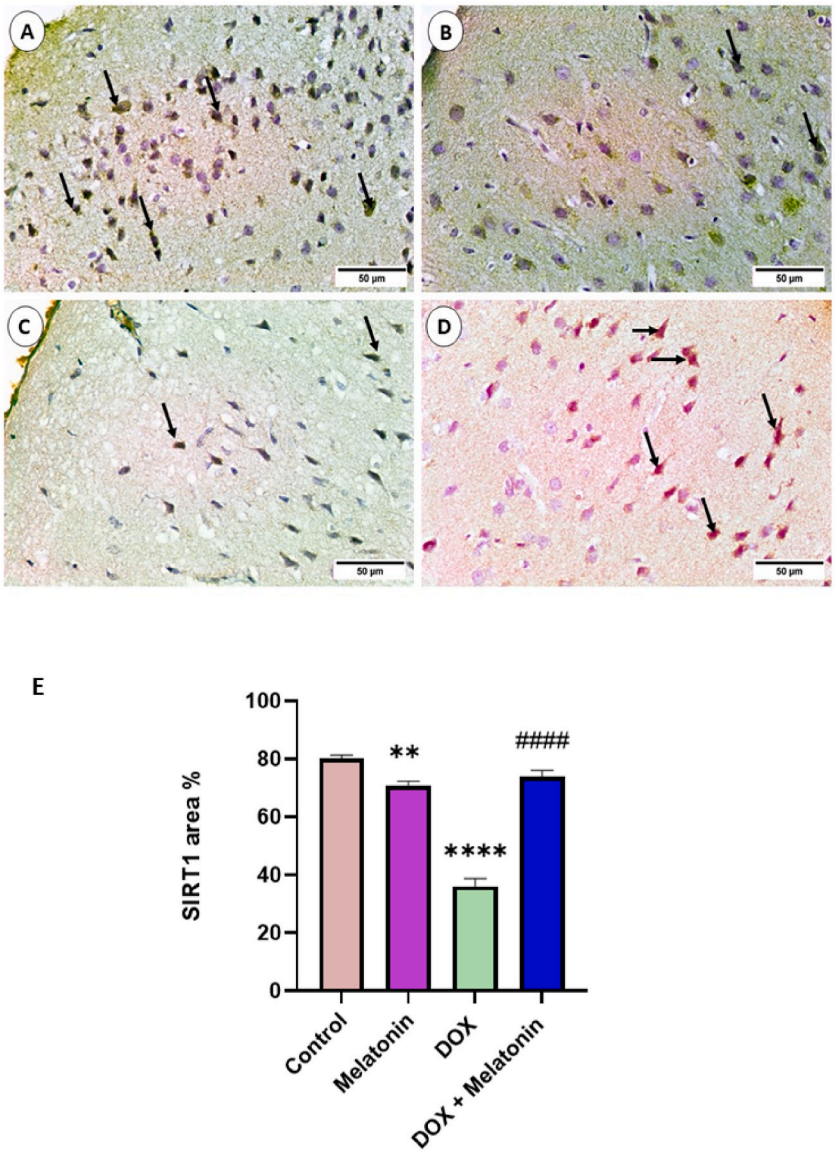


Fig. 5. Photomicrograph of SIRT1 immunostaining in the PFC sections.
(A), (B): Control and melatonin-treated groups showed strong positive SIRT1 reaction (arrows). **(C)** The DOX-treated group revealed a weak positive SIRT1 reaction. **(D):** Combined melatonin and doxorubicin-treated group showed moderate positive SIRT1 reaction **(**SIRT1 x 400). **(E):** Area percentage of SIRT1 revealed a statistical reduction in DOX treated group compared to the control ($P < 0.0001$), which is reversed after cotreatment with melatonin.

3.4. Immunohistochemical staining of Nrf2, p53 and SIRT1 in the PFC sections

Control and melatonin-treated groups demonstrated a strong positive Nrf2 immunostaining in the prefrontal cortex region (Fig. 3A and B). However, the Dox-treated group showed weak positive Nrf2 reaction (Fig. 3C). The combination of melatonin and Dox showed a strong positive Nrf2 reaction (Fig. 3D). There was a magnificent minimization in the Nrf2 stained area percentage in the Dox treated group as compared to the control ($P < 0.0001$), however, it increased significantly after co-treatment with melatonin ($P < 0.01$) as compared to the Dox group (Fig. 3E).

The p53 marker demonstrated weak reaction in control and melatonin groups (Fig. 4A and B). However, the Dox group displayed a strong positive p53 immuno-staining (Fig. 4C). Moreover, the combined melatonin and Dox group revealed moderate positive p53 stain (Fig. 4D). A significant increase in p53 stained area intensity percentage in the Dox group compared to the control, while it downregulated significantly in the melatonin/Dox co-treated group as compared to the Dox group ($P < 0.0001$) (Fig. 4E).

The control and melatonin-treated groups showed a strong positive SIRT1 immunostaining (Fig. 5A and B). However, the Dox-treated group revealed a weak positive SIRT1 reaction (Fig. 5C). Co-treatment with melatonin and DOox demonstrated a moderate positive SIRT1 reaction (Fig. 5D). SIRT1 stained area percentage revealed a statistically significant diminution in the Dox group compared to the control ($P < 0.0001$), and statistically elevation in the co-treated group as compared to the Dox-treated group ($P < 0.0001$) (Fig. 5E).

3.5. mRNA quantification of Nrf2, SIRT1 and p53 genes in the PFC

mRNA levels of Nrf2, SIRT1 and p53 (normalized against GAPDH, $n = 4$) were investigated by qRT-PCR analysis and demonstrated significant differences in the fold changes among the experimental groups. Dox treatment downregulated NRF2 ($P < 0.01$), SIRT1 ($P < 0.05$), mRNA expression levels compared to the control (Fig. 6B and F), however it upregulated p53 ($P < 0.0001$), compared to the control (Fig. 6D). Whereas cotreatment with melatonin elevated mRNA expression levels of Nrf2, and SIRT1 significantly ($P < 0.05$), and reduced p53 expression compared to Dox-treated group ($P < 0.05$) (Fig. 6B–D and F).

3.6. Nrf2, p53 and SIRT1 protein levels by western blotting analysis

Western blot study ($n = 4$) of Nrf2, p53 and SIRT1 protein levels in the control, melatonin, Dox and co-treated groups showed that Nrf2 protein level downregulated significantly in the Dox-treated group as compared to the control ($P < 0.0001$), and upregulated significantly in the melatonin/Dox co-treated group as compared to Dox-treated group ($P < 0.05$), whereas Dox treatment showed a significant upregulation in p53 protein values (normalized against β-Actin) compared to the control ($P < 0.0001$), whereas the co-treated group displayed a significant downregulation of p53 protein compared to Dox treated group ($P < 0.05$). Statistical analysis also revealed a significant downregulation in SIRT1 protein level as compared to the control ($P < 0.01$), and a significant elevation in the co-treated group as compared to the drug treated ($P < 0.05$) (Fig. 7). These intraspecific differences corresponded with area percentage changes seen by immunohistochemical morphometric analysis.

4. Discussion

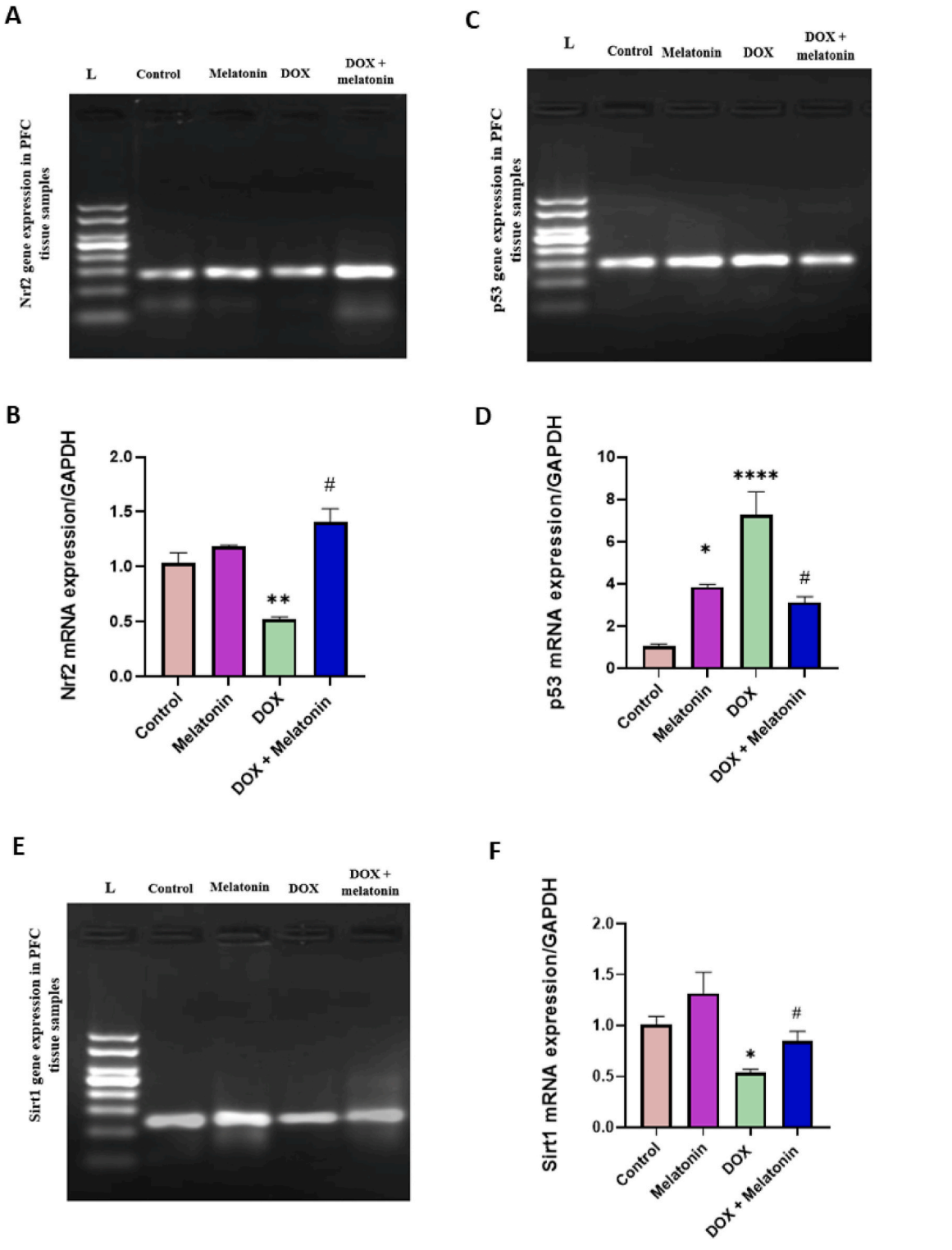
Chemotherapy could directly or indirectly affect the brain function [45,46]. Dox is one of the most widely used chemotherapy in breast cancer treatment [47]. Patients treated with Dox showed poor cognitive skills or chemo brain [48,49]. In our study, we explored the underlying mechanisms behind Dox induced chemo brain and the potential protective role of melatonin on the affected brain. We found that treatment with melatonin inhibited Dox-induced neurotoxicity in neurobehavioral evaluation test, oxidative stress markers measurements, prefrontal cortex and hippocampal histopathological assessments, mRNA and protein levels of Nrf2, p53 and SIRT1 markers quantifications.

First, the spatial memory was examined in the rat model before scarification, then the oxidative stress/antioxidant enzyme, beside the expression levels of Nrf2, p53 and SIRT1 markers by various cellular and molecular mechanisms were also investigated. The data revealed that the Dox induced memory problem and caused spatial memory deficit, which was reversed after co-treatment with melatonin.

Results of our study agreed with previous studies concluded that Dox decreased the spatial memory abilities relative to the control groups [50–52]. Melatonin also improved the cognitive performance in breast cancer patients receiving chemotherapy in a randomized control study [53], however, the underlying mechanism behind its neuroprotective effect is still unknown.

Our study confirmed that Dox treatment significantly persuaded NO oxidative stress marker, which was lessened by co-treatment with melatonin. However, the opposite occurred in the antioxidant enzyme GSH, where there was a significant decrease in Dox treated rats and an increase following the co-treatment with Dox and melatonin. These findings agreed with [54] who found that NO production was noticed to increase in the brain tissues of Dox-treated mice. However, GSH levels were observed to decrease in DOX-treated rats, which was restored by melatonin [19,34,51,55]. Dox was also noted to significantly decreased cardiac GSH in rats [56]. Dox lipid peroxidation induction is an indicator of brain oxidative stress [57]. Melatonin is known for its antioxidant properties [58]. It can prevent the oxidative stress generated neuronal damage by Dox through antioxidant enzymes activation and thus increasing neurogenesis.

The histopathological results of the current study displayed evident brain damage in the Dox treated rats. It showed disorganized deeply stained pyramidal neurons and pyknotic nuclei in the hippocampal sections, indicating degeneration, compared to tiny



(caption on next page)

Fig. 6. Relative quantitation measurement of the studied genes in rat brain by RT PCR and gel electrophoresis.
(A): Nrf2 qPCR product (121 bp). **(B)**: Data were presented as mean ± SEM of Nrf2 fold changes, where Nrf2 showed significant downregulation in the Dox group compared to the control ($P < 0.01$) and was upregulated significantly in the melatonin co-treated group compared to the DOX group ($P < 0.01$). **(C):** p53 qPCR product (146 bp). **(D)**: Data were presented as mean ± SEM of the fold changes of the p53 gene. where p53 displayed significant upregulation in the Dox group compared to the control ($P < 0.0001$) and significant downregulation in the melatonin co-treated group compared to the DOX group ($P < 0.05$). **(E)**: Sirt1 qPCR product (137 bp). **(F)**: Data were presented as mean ± SEM of Sirt1 fold changes, where Sirt1 demonstrated significant downregulation in the Dox group compared to the control ($P < 0.05$) and upregulate significantly in the melatonin co-treated group compared to the DOX group ($P < 0.05$). (B-D-F) p-value specified significantly by One-Way ANOVA and unpaired *t*-test. The original gel was provided as supplementary material.

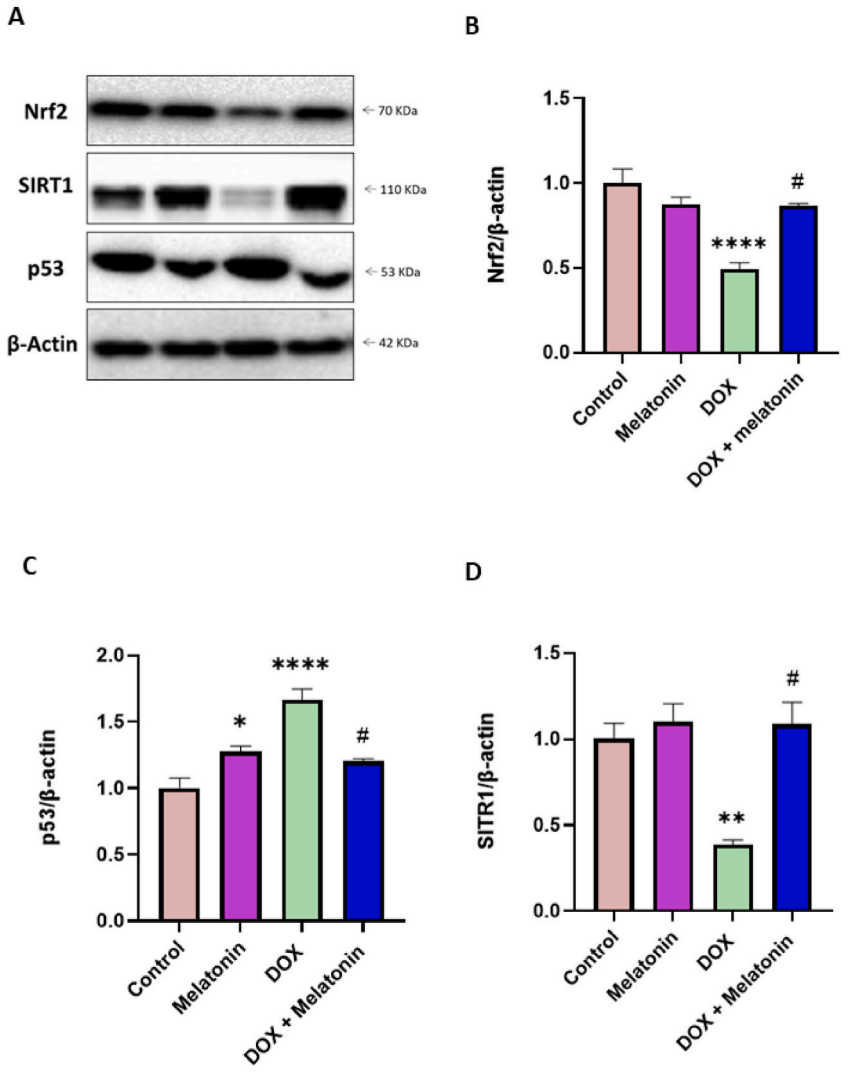


Fig. 7. Representative western blotting and histograms showing protein expression of Nrf2, p53 and SIRT1.
(A): Representative western blot showing protein expression of Nrf2, p53 and SIRT1. The mean optical density ± SEM of normalized Nrf2, p53 and SIRT1 expression of control melatonin, DOX treated and cotreated groups, show that there was significant downregulation of Nrf2 expression in the DOX group as compared to the control ($P < 0.0001$). However, it was upregulated significantly after cotreatment with melatonin compared to the DOX-treated group ($P < 0.05$) **(B).** For p53, the DOX-treated group showed significant upregulation compared to the control ($P < 0.0001$). Where it was downregulated in the DOX-melatonin cotreated compared to the DOX treated ($P < 0.05$) **(C).** However, SIRT1 was significantly reduced in the DOX compared to the control ($P < 0.01$) and was upregulated in the cotreated group as compared to the Dox group ($P < 0.05$) **(D).** One-way ANOVA was utilized to compare all the groups, and an unpaired *t*-test was applied to compare each group.
Control images were re-used for illustrative purposes. The original blot was provided as supplementary material.

pyramidal neurons with vesicular nuclei and prominent nucleoli in the control groups. Moreover, it displayed numerous deeply shaded neurons with pyknotic nuclei surrounded by halos and typical microglial cells indicating focal gliosis in the prefrontal cortex sections of Dox treated rats compared to normal appearing granule cells with rounded vesicular nuclei and prominent nucleoli in the control groups. Nevertheless, the co-treated group with melatonin showed that the pyramidal neurons mostly with vesicular nuclei and prominent nucleoli, while few deeply stained neurons surrounded by halos were still present in the hippocampal sections. Moreover, in the prefrontal cortex sections, the melatonin/Dox co-treated group also demonstrated restoration of the prefrontal architecture. Pyramidal and granule cells appeared normal. While some deeply shaded neurons with pyknotic nuclei adjoining by halos were still displayed as well. This indicates the adverse outcome of Dox on the brain tissue and the protective role of melatonin against Dox neurotoxicity.

Dox in other studies, also induced focal gliosis in the prefrontal cortex and degeneration in the hippocampus of the treated rats [51, 59]. Melatonin co-administration in a methotrexate treated rat, induced neurogenesis in the hippocampus [60].

To further evaluate in depth the underlaying mechanisms behind the Dox induced neurotoxicity and the protective effect of melatonin, NRF2, p53 and SIRT1 markers were examined by immunohistochemistry, gene expression and western blotting techniques.

For Nrf2, the immunohistochemical results revealed that the Dox-treated group showed weak positive Nrf2 immunostaining compared to the control and melatonin-treated groups, which demonstrated a strong positive Nrf2 stain in the prefrontal cortex region. However, the combination of melatonin and Dox showed a strong positive Nrf2 reaction. There was also a magnificent reduction in the Nrf2 stained areas' percentage in the Dox treated group compared to the control, which was increased significantly after the co-treatment with melatonin compared to the Dox group. This was concomitant with the gene expression and the western blotting analysis findings, where Nrf2 mRNA expression and protein level in the Dox treated groups demonstrated a significant downregulation as compared to the control and upregulated significantly in the melatonin/Dox co-treated group compared to Dox treated.

These findings were concomitant with [49] who found that Dox induced chemo brain was associated with the reduction of Nrf2 protein levels in the rats' hippocampal tissues. melatonin has been also shown to have a neuroprophylactic effect against metal chelation through upregulating Nrf2 signaling [61]. Melatonin significantly raised Nrf2 translocation to the nucleus, significantly upregulated expression of Nrf2 genes, and increased the level of Nrf2 protein expression in the hippocampus of lipopolysaccharide prompted the mice's depressive like behaviour. Moreover, siRNA mediated Nrf2 knockdown altered melatonin's sequelae on pyroptotic cell arrest and ROS production [25].

Moving to p53 marker, the current study demonstrated a strong positive immunostaining in the Dox group as compared to the weak positive reaction in the control and the melatonin-treated groups. Moreover, the combined melatonin and Dox-treated group showed moderate positive p53 stain. There was also a notable increase in the intensity of the p53 area percentage in the Dox treated group as compared to the control, and a significant reduction in the co-treated group as compared to the Dox group. These findings matched the gene expression and the western blotting results, where p53 mRNA expression and protein level in the Dox treatment also revealed a significant upregulation as compared to the control, while the co-treated group showed a significant downregulation compared to Dox treated group.

These findings were explained by Ref. [62] who reported that Dox promoted cell death by activating p53. Suppression of p53 attenuated Dox prompted cell damage [63,64].

Turning to SIRT1 marker, the current study revealed that the Dox treated group showed a weak SIRT1 immunostaining compared to a strong positive SIRT1 reaction in the control and melatonin-treated groups. However, co-treatment with melatonin and Dox demonstrated a moderate positive SIRT1 reaction. SIRT1 area percentages revealed a significant reduction in the Dox treated group as compared to the control, and an elevation in the co-treated group as compared to the Dox group. The SIRT1 mRNA quantification and western blotting analysis revealed also a significant reduction in SIRT1 protein value and mRNA expression compared to the control, and a significant elevation in the co-treated group compared to the Dox-treated group. The results of the current study were explained by various previous studies that found bi-directional crosstalk connecting SIRT1 and Nrf2 in renal mesangial cells [65]. Nrf2 nuclear localization ameliorated by SIRT1 via p53 inhibition in mesenchymal stem cells [66]. SIRT1 activated AMPK phosphorylated and Nrf2 in macrophage cells [67]. Leptin prompted Nrf2 activated SIRT1 bonding to ARE location in SIRT1 advocator [68]. Melatonin was associated with SIRT1 signaling activation in microglia and ameliorated LPS-induced acute depressive like behaviour [25].

Therefore, our results suggested that Nrf2 displayed a vital role in the pathogenesis of Dox induced chemo brain and the protective role of melatonin was displayed via regulating p53 and SIRT1. This study was the first to denote a functional link between Nrf2/p53 and SIRT1 expression in the mechanism behind neuroprotective role of melatonin against chemo brain induced by Dox treatment. The current study elucidated the molecular mechanisms implied in the pathogenesis of Dox-chemo brain by Nrf2 via p53 and SIRT1. Our findings present mechanistic insight into how Nrf2 contributes to the alimentation of the brain's functional stability during chemotherapy. Moreover, Nrf2 nuclear localization preservation is an essential target for the avoidance of neuronal injury and spatial memory deficiency during chemotherapy course through melatonin supplementation.

Further studies are essential to completely figure out the effect of Dox on other brain regions. Also, clinical studies would be of a great benefit to confirm our hypothesis that melatonin is an effective supplement to be added to Dox chemotherapy regimen to protect against Dox hazardous effects on the brain.

5. Conclusions

The current study featured the drawbacks of Dox on spatial memory, which were firmly associated with oxidative stress aggravation, activation of p53, and suppression of Nrf2 and SIRT1 expressions in the hippocampus and PFC. Moreover, the data highlighted the beneficial role of melatonin in memory restoration and brain cellular damage repair via mitigating brain oxidative stress,

promoting neurogenesis and modulating Nrf2/p53 and SIRT1 signaling pathways. Thus, melatonin could be an adjuvant supplement to the Dox regimen for cancer patients.

Ethical approval

All animal experiments were complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. It was authorized by Mansoura University Animal Care and Use Committee (MU-ACUC) (Code number: VM. R.23.07.111).

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Data availability

Data will be made available on request.

CRediT authorship contribution statement

Neven A. Ebrahim: Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Mohamed R. Elnagar:** Writing – review & editing, Methodology. **Randa El-Gamal:** Writing – review & editing, Methodology. **Ola Ali Habotta:** Writing – review & editing, Supervision. **Emad A. Albadawi:** Writing – review & editing, Funding acquisition. **Muayad Albadrani:** Writing – review & editing, Funding acquisition. **Abdulrahman S. Bahashwan:** Writing – review & editing, Funding acquisition. **Hend M. Hassan:** Writing – review & editing, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38081.

Abbreviations

Dox	doxorubicin
NRF2	nuclear factor erythroid 2-related factor 2
SIRT1	Silent information regulator 2 homolog 1
PFC	prefrontal cortex
NO	Nitric Oxide
GSH	reduced glutathione
TNFα	tumor necrosis factor alpha
ROS	Reactive oxygen species
NF-E2	nuclear factor-erythroid 2
NF-κB	nuclear factor kappa
H&E	hematoxylin and eosin
PBS	phosphate buffered saline
BSA	bovine serum albumin
DAB	Diaminobenzidine
qRT-PCR	quantitative reverse transcription-PCR
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
CA1	Cornu Ammonis 1

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