The possible protective role of melatonin and exosomes derived from Mesenchymal stem cells on cisplatin induced testicular injury in adult male albino rats: Histological and Immunohistochemical study.

Abstract

Overveiw: Cisplatin (CIS) is a perfect antineoplastic medicine resulting in severe testicular toxicity. Melatonin is a potent antioxidant that can prevent gonadal toxicity. exosomes have been demonstrated to have a vital effect in tissue regeneration.

Aim: to evaluate the potential protective effects of melatonin and exosomes developed from bone marrow mesenchymal stem cells (BMSC-EX) in testicular toxicity caused by cisplatin.

Materials and Methods: fifty rats were divided in a random way into five equal groups. Group I (control group). Group II were given a single dose (8 mg/kg) of cisplatin intraperitoneally I.P. Group III (melatonin group): intra peritoneal administration of melatonin (4 mg/kg) started 5 days before cisplatin injection for 15 days. Group IV (exosomes group): a single intra venous dose of 100 µg BMSC-EX one day before cisplatin dose. Group V (melatonin and exosomes). Specimens of the testicles were collected and processed for immunohistochemical and histological analysis.

Results: Group II declared distortion, irregular outline of the seminiferous tubule, with critical accumulation ($P < 0.01$) of collagen fibers. A substantial elevation was present ($P < 0.01$) in TNF-α immunostaining and substantial reduction ($P < 0.01$) was seen in AR and PCNA immunostaining in comparison to control group. Groups III and IV exhibited some microscopic histological variations development, a critical reduction ($P < 0.01$) was also observed in collagen fibers deposition and TNF-α immunostaining and critical elevation ($P < 0.01$) was present in AR and PCNA immunostaining in comparison to group II. In comparison to control group, group V histological structure was similar.

Conclusion: Each of melatonin and exosomes can protect against testicular toxicity caused by cisplatin when administered before and during cisplatin therapy, but their combined administration has better results.

Key Words: Cisplatin, Melatonin, Exosomes.

INTRODUCTION

Chemotherapy is considered the principal way for cancer therapy. Although it is an effective treatment, it is limited because of its toxic effects on organs[1]. Infertility is one of the most common toxic effects caused by chemotherapeutic agents[2].

Cisplatin (CIS) is an effective antineoplastic prescription abused in the cure of distinctive types of tumors such as neoplasms, lung, colorectal, hematologic, ovarian, and testicular cancer. The successful use of CIS has been hampered by conventional toxicities such as nephrotoxicity, hepatotoxicity, neurotoxicity and cardiotoxicity[3].Testicular dysfunction is the most reported consequence resulting from exposure to cisplatin[4].

Melatonin, which is emitted from the pineal gland has a powerful antioxidant effect and the capability of scavenging free radicals. Melatonin induces multiple antioxidant enzymes and inhibits pro-oxidant enzymes, so it protects cellular components against oxidative damage. Further, melatonin enhances the repairing processes of DNA damage resulting from chemotherapeutic agents by stimulating different repair enzymes[5].

Exosomes are one of extracellular vesicles approximately 100 nm in size (diameter of 40–150 nm). MSC-Exosomes which have different types of proteins, nucleic acid, abundant miRNAs

and lipids can transmit them into cells and from one cell to another type of cell enhancing intercellular communication, immunoregulation, metabolism and damage repair[6].

This research was performed to evaluate the potential protective effects of the antioxidant drugs Melatonin and mesenchymal stem cells derived exosomes in testicular toxicity induced by cisplatin.

MATERIALS AND METHODS

Drugs & Chemicals

Cisplatin

Produced by Mylan, France, and acquired in vial form from Pfizer Chemical Company, USA. Every infusion concentrate vial includes: 1 mg/mL CIS, hydrochloric acid, and sodium hydroxide to about 4.0 pH, 9 mg/mL sodium chloride and water for injection to 50 mL or 100 mL for final volume. In the present study, a single I.P. (8 mg/kg) of cisplatin was administered [7].

Melatonin

Melatonin in tablets (3 mg) was utilized in this research. P. P., INC (USA). Sigma Chemical factory supplied them (S.t. Louis; Missouri, USA). Capsules were broken up, diluted in distilled water, and administered intraperitoneally at 4 mg/kg/day dosage for ten days [8].

Exosomes and their preparation from MSCs

The supernatant of MSCs was used to extract exosomes generated by mesenchymal stem cells. First, the central lab of Cairo University's stem cell and molecular biology section created rat BM-MSCs. In Dulbecco's Modified Eagle Medium (DMEM) containing 0.5 percent human serum albumin (HSA), MSCs were grown (Sigma-Aldrich, St. Louis, MO, USA). Trypan blue exclusion was used to determine that the cultivated cells were more than 99 percent viable. After being plated at a density of 4,000 cells per cm2 for seven days, the cells were trypsinized, counted, and replated in expansion medium at a density of 2,000 cells per cm2 for an additional seven days. The cells were then stored at -80 °C, centrifuged twice, once at 2,000 g for 20 minutes to remove debris and once more at 100,000 g for an hour at 4 °C (Beckman Coulter, Fullerton. For this test, exosomes were kept at -80 degrees Celsius [9].

Rats were injected with BMSC-EX as 100 µg intravenous solitary dose diluted in 0.5 ml of PBS[10].

Animals and Diet:

At this research, **60** adult healthy albino male rats about 180 -200 grams weight were employed. The animals were supplied from the veterinary school's animal shelter at Moshtohor, Benha University. Animals were housed in suitable cages under the current climatic conditions with intense care and good procedures to maintain their normal health. They continued to consume a typical, balanced diet and just tap water. The animal facilities made sure that all moral guidelines for treating animals were fulfilled. The Institutional Animal Care Committee at Benha University, Egypt, authorized the work protocol. {M.D. 2.6.2020}

Experimental design:

This study was done from November 2021 to December 2021. 60 male adult albino rats were separated after one week of housing as follows:

10 rats were used to isolate the exosomes from the bone marrow mesenchymal stem cells.

The rats (50) were split to five groups. **(n= 10).**

Group I (control group): divided into five equal subgroups (n=2):

Ia Subgroup: no treatment.

Ib Subgroup: took a daily dose of 0.9% sodium chloride as cisplatin vehicle for ten days.

Ic Subgroup: took a daily dose of distilled water as melatonin solvent for ten days.
Id Subgroup: were given distilled water for five days followed by ten days of distilled water and 0.9 percent sodium chloride
Subgroup Ie: 2 rats were given an intravenous injection of PBS (0.5 ml) as a single dose of exosomal vehicle.

Group II (cisplatin group): The rats were kept up to four weeks after receiving a single dose (8 mg/kg) of cisplatin I.P.

Group III (melatonin group): CIS as in group II and intraperitoneal melatonin (4 mg/kg) were given to rats before the single intraperitoneal dose of cisplatin, melatonin therapy began five days earlier. It then continued for an additional ten days.

Group IV (exosomes group): rats received a single intra venous BMSC-EX 100 µg dose diluted in 0.5 ml PBS and on the next day received a single dose of cisplatin as in group **II**.

Group V (combined melatonin and exosomes group): rats received a single intravenous BMSC-Exos dose as in group **IV**, melatonin as in group **III** and a single dose of cisplatin as in group **II**.

Sampling:

The rats were given ether anaesthesia before being cervically decapitated after four weeks from cisplatin injection then Specimens of the testes were collected and fixed for 24 h in formalin 10% following that it was refined and paraffin-embedded. For routine, special staining, and immunostaining, serial slices of 5-7 µm thickness were cut.

Histological and Immunohistochemical Studies

5-7 m thick paraffin slices were placed on glass slides for H&E staining and Masson trichrome staining to show the accumulation of collagen fibers. For immunohistochemical staining, additional slices were put on +ve charged slides[11].

Immunohistochemical staining for Proliferating Cell Nuclear Antigen **(PCNA)**, androgen receptor **(AR)** recognition &Tumor necrosis factor alpha **(TNF-α)**. The rabbit polyclonal antibody was the primary antibody employed (Lab Vision Corporation, Neomarkers Laboratories, Westinghouse, Thurmont, California, USA). Deparaffinized and hydrated paraffin slices were used. The slices were treated with primary antibodies after suppressing the endogenous peroxidase activity with 10% hydrogen peroxide. The secondary antibody was then used following phosphate buffer washing (biotinylated goat anti rabbit). Avidin-biotin peroxidase, which binds to the biotin on the secondary antibody, was incubated on the slides. After adding (diaminobenzidine) chromogen, which peroxidase turns into a brown precipitate, the site of antibody binding was made visible. Meyer's hematoxylin was used to counterstain various sections. As a negative control, the primary antibody was replaced with phosphate-buffered saline (PBS). The reaction site was the brown coloration of nuclei in AR and PCNA and brownish color of cytoplasm in TNF-α [12].

Morphometric study

All immunological parameter's mean area % of collagen fiber accumulation, PCNA, and TNF-α immuno-expression were assessed in 10 sections from 10 non-overlapping fields of each rat in all groups by version 6.0. of program Image-Pro Plus (Media. Cybernetics. Inc., Bethesda, Maryland., USA). The Benha University's Microscopic Photography Unit used a Leica DM2500 optical microscope, with a magnification power [X400], to capture the digital photos (Histology Department, Faculty of Medicine).

Statistical analysis

The IBM SPSS statistics software for windows, version 26 was utilized to collect and analyze all the experiment's data (IBM Corp., Armonk, NY, USA). To compare variations in the

groups of morphometric outcomes, ANOVA with a Post Hoc LSD test was utilized. Data plotted as standard deviation (SD), mean (M) and changes were deemed substancial for each test at $P < 0.01$.

RESULTS

MSCs- Exosomes Characterization:

Purified exosomes underwent transmission electron microscopy analysis, which revealed double membrane bound spherical form (40-100 nm) (figure 1).

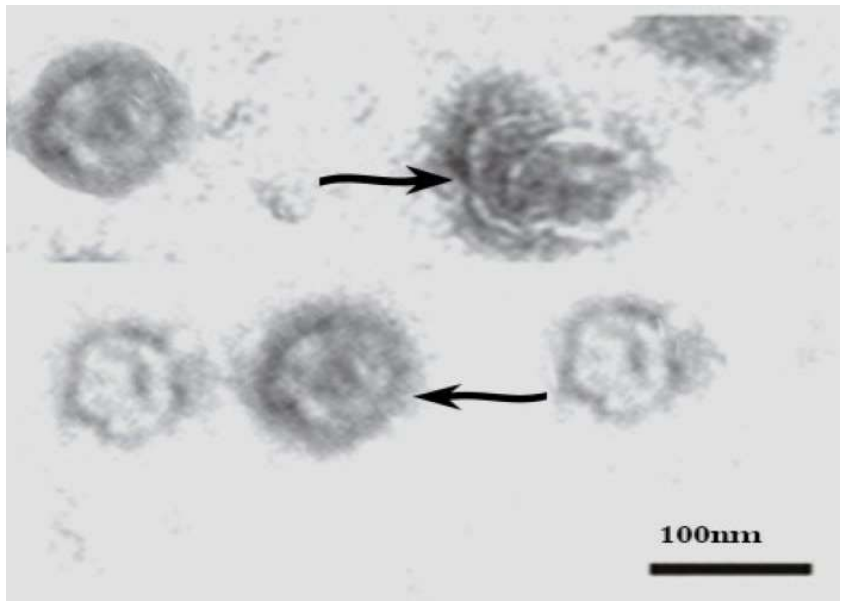


Fig. (1): A transmission electron micrograph of exosomes reveales their distinctive 40–100 nm-diameter spheroid double-membrane bound shape (black arrow). **(X2000)**

H & E Stain results:

Similar histological architecture was seen in all subgroups of group I (the control group). Sections revealed the normal histological structure of testis formed of closely packed seminiferous tubules surrounded by thin basement membrane (BM), had regular outline, loose vascular interstitial tissue in between containing Leydig cells. Every tubule had a germinal epithelium resting on a BM that contained spermatogenic cells in various stages of development. Proliferating germ cells such as spermatogonia, both dark and pale types, primary spermatocytes, spermatids forming multiple layers, and spermatozoa were found in the germinal layer. Sertoli cells lined the tubules as well, (figure 2a1&a2).

Group II (cisplatin group): revealed that most STs have distorted shapes and uneven edges with apparent decrease in the germinal epithelium thickness. Detachment of the BM with relative widening of interstitial spaces with dilated congested interstitial blood vessels (BVs). Most of the primordial spermatocytes had deeply acidophilic cytoplasm and darkly stained nuclei. Spermatids were irregularly dispersed with few sperms, (figure 2b).

Group III (melatonin group): displayed a regular contour of the majority of the STs lined with the various stages of spermatogenic cells but still some areas lose spermatogenic cells. Tails of mature sperms were also visible extending in the lumen of the tubules. Wide interstitial tissue with a discontinuity of BM and congested blood vessels (figure 2c).

Group IV (exosomes group): The majority of the STs in group IV's testis had a regular outline, filled with germinal epithelium with wide interstitial tissue. Most spermatogonia had initial spermatocytes with darkly black stained nuclei that were arranged in one or two layers, followed by many layers of spermatids with mature sperm tails that protruded into most of the tubules (figure 2d).

Group V (combined melatonin and exosomes): revealed the regular outline of STs lined with germinal lining with full spermatogenesis but there was slight reduction in some tubules, the interstitial tissue displayed more Leydig cells (figure 2e).

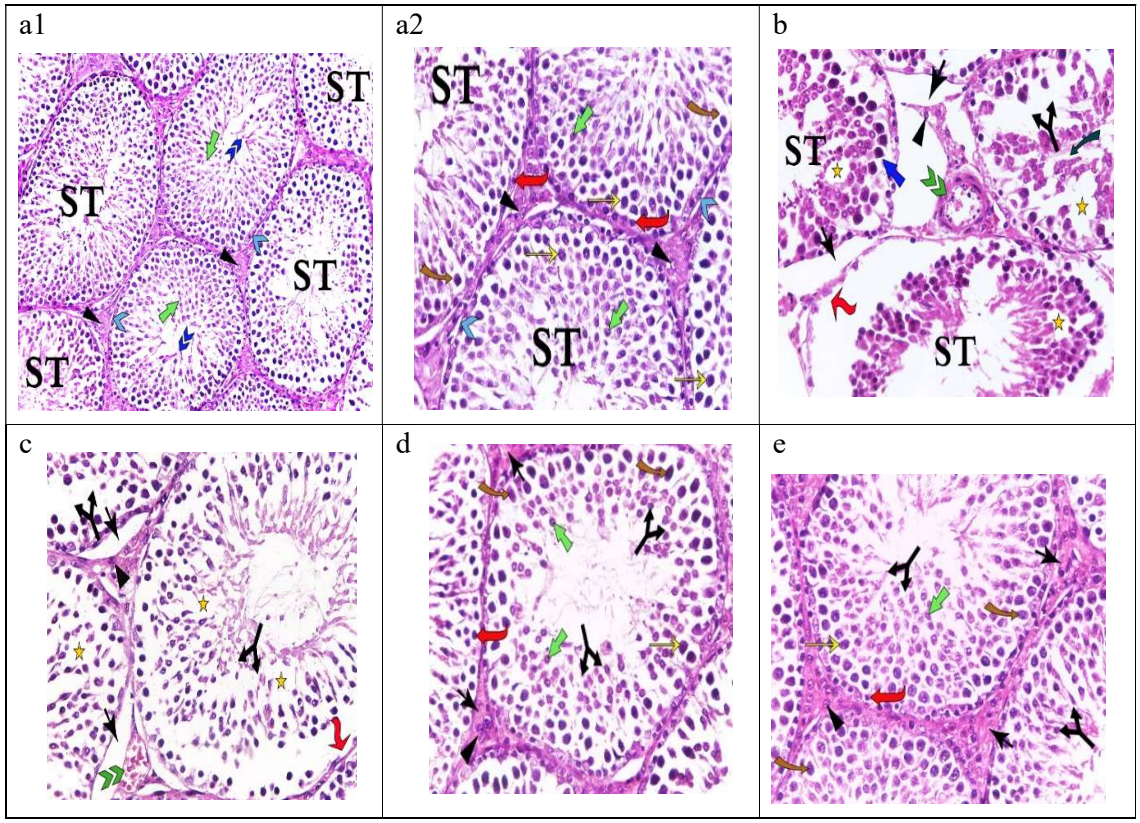


Figure 2: (a1) A photomicrograph of a section in testis of a rat in group I (**control group**) declares STs with regular outline and average BM ( ), numerous spermatogenic cells layers can be seen in the tubules ( ) with lumina containing sperms ( ), Leydig cells are present in the loose vascular interstitial tissue that surrounds the tubules **( ). [H&E, X200] (a2)** showing A higher magnification of the previous photomicrograph demonstrating the ST's typical structure with regular outlines and average BM ( ). Spermatogonia can be seen in the germinal layer ( ), Sertoli cells ( ), primary spermatocytes ( ) and spermatids ( ). A loose vascular interstitial tissue containing leydig cells surrounds the tubules ( ). **(b) group II (cisplatin group)** showing most STs (**ST**), have distorted and uneven shapes, detached BM ( ), marked reduction in germ cell lining ( ), wide interstitial tissue ( ) with blood vessels congested ( ). vacuolations are found inside the tubules ( ), primordial spermatocytes had deeply acidophilic cytoplasm and darkly stained nuclei ( ), irregularly dispersed Spermatids ( ) and decreased

Leydig cells (). **(c) group III (melatonin group)** showing minimal changes with regular outline of most of the STs with slightly thickened BM (), slightly reduced germinal lining (). Inside the tubules, vacuolations are found (). There are large interstitial spaces between tubules () with blood vessels congested () and Leydig cells (). **(d) group IV** (exosomes group) showing most STs have a consistent shape and contain spermatogenic cells at various stages (); spermatogonia (), primary spermatocytes (), spermatids () and Sertoli cells (). Loose vascular tissue fills between gaps (). and Leydig cells (). **(e) group V** (combined melatonin and exosomes) showing STs with regular outline, average BM, germinal lining formed of spermatogenic cells multiple layers (); spermatogonia (), primary spermatocyt (), spermatids () and Sertoli cells () with lumina containing sperms and average interstitial tissue () with Leydig cells (). **[H&E, X400]**

Masson trichrome staining results:

Group I (control): demonstrated the typical distribution of fine collagen fibres at the STs boundary, surrounding BVs (figure 3a). while the cisplatin group showed intense perivascular accumulation of dene collagen fibers in blood vessels wall, basement membrane of STs and in between the tubules, (figure 3b).

Melatonin, exosomes & combined melatonin groups showed decreased deposition of fine collagen fibers in the wall of BVs and the seminiferous tubules BM, (figure 3c, 3d, &3e).

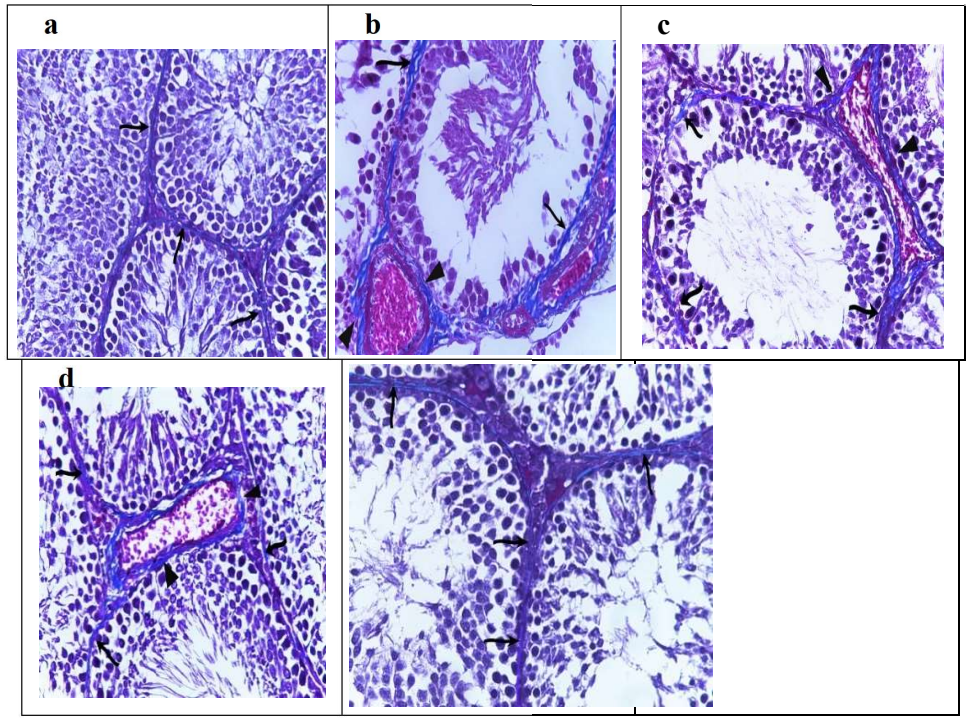


Figure 3: A Photomicrograph of a section in the testis of a rat from the **(control group)** shows fine collagen fibers between and at the margin of STs, surrounding BVs (). **(b) (cisplatin group)** shows dene collagen fibers markedly deposited in BVs wall (), seminiferous tubules BM and in between them (). **(c) (Melatonin group)** shows fine collagen fibers moderately deposited in the blood vessels wall () and the BM of seminiferous tubules (). **(d) (Exosomes group)** shows mild deposition of fine collagen fibers the wall of BVs () & seminiferous tubules BM (). **(e) (Melatonin and exosomes**

group) shows a minimal deposition of fine collagen fibers in the BM of STs (⤳). **(Masson trichrome staining X400)**

Androgen Receptor (AR) staining results:

Group I: revealed +ve AR immunoreactivity in the nuclei of Leydig interstitial cells, the Sertoli cells, myoid cells surrounding the STs. Spermatogenic cells bordering the STs exhibited negative immunoreactivity (figure 4a). while the cisplatin group indicated that spermatogenic cells, myoid cells, leydig cells, and Sertoli cells all had -ve nuclear AR immunoreactivity (figure 4b).

Melatonin, exosomes, and combined exosomes & melatonin group showed more expression of positive anti-AR immune reactivity in some Sertoli cells, myoid cells and Leydig cells, (figure 4c, 4d & 4e).

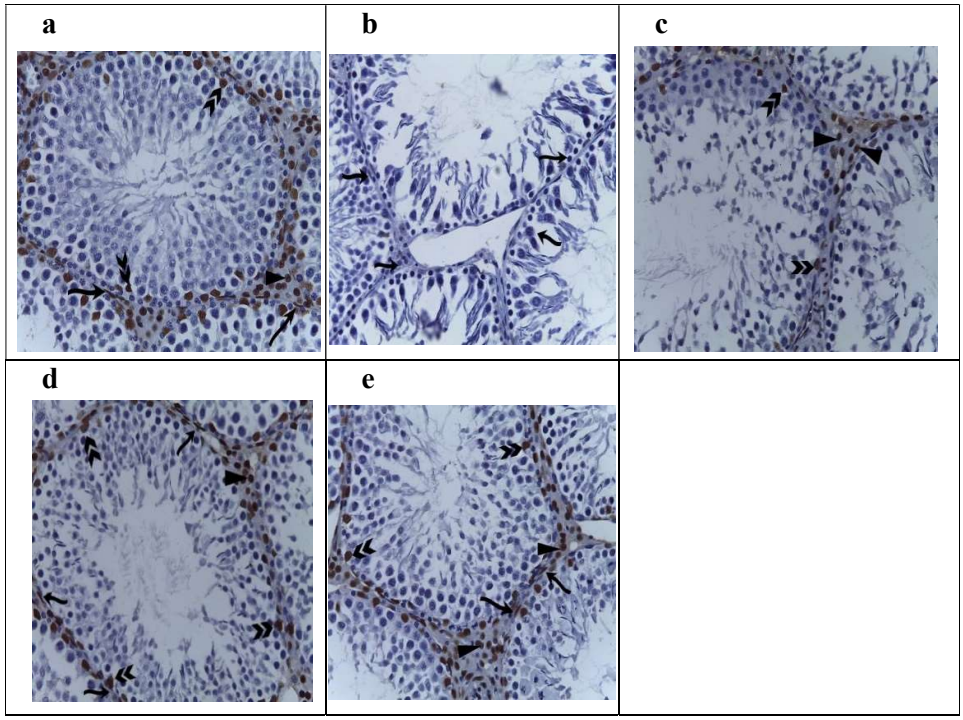


Figure 4: A Photomicrograph of a section in the testis of a rat from the **(control group)** reveals +ve AR immunoreactivity in the myoid cell's nuclei **(⤳),** both in and around the Sertoli cells (🡅) and Leydig cells (◣). **(b) (cisplatin group)** reveals negative AR immune_ expression (⤳). **(c) (melatonin group)** reveals that few of Sertoli cells (🡅) and few Leydig cells (◣) display +ve nuclear AR immune expression. **(d) (exosomes group)** reveals +ve AR immune expression of some Sertoli cells (🡅), Leydig cells (◣) and myoid cells (⤳). **(e) (melatonin and exosomes group)** reveals that myoid cells **(⤳)**, in addition to Sertoli cells (🡅) and Leydig (◣) display strong +ve AR immune expression. **(Anti AR, X400)**

Proliferating Cell Nuclear Antigen (PCNA) staining results:

Control group showed positive PCNA immune reactivity, (figure 5a) while in cisplatin group showed negative immune reactivity of PCNA, (figure 5b). Melatonin, exosomes, and combined

exosomes & melatonin group showed more expression of positive PCNA immune reactivity, (figure 5c, 5d &5e).

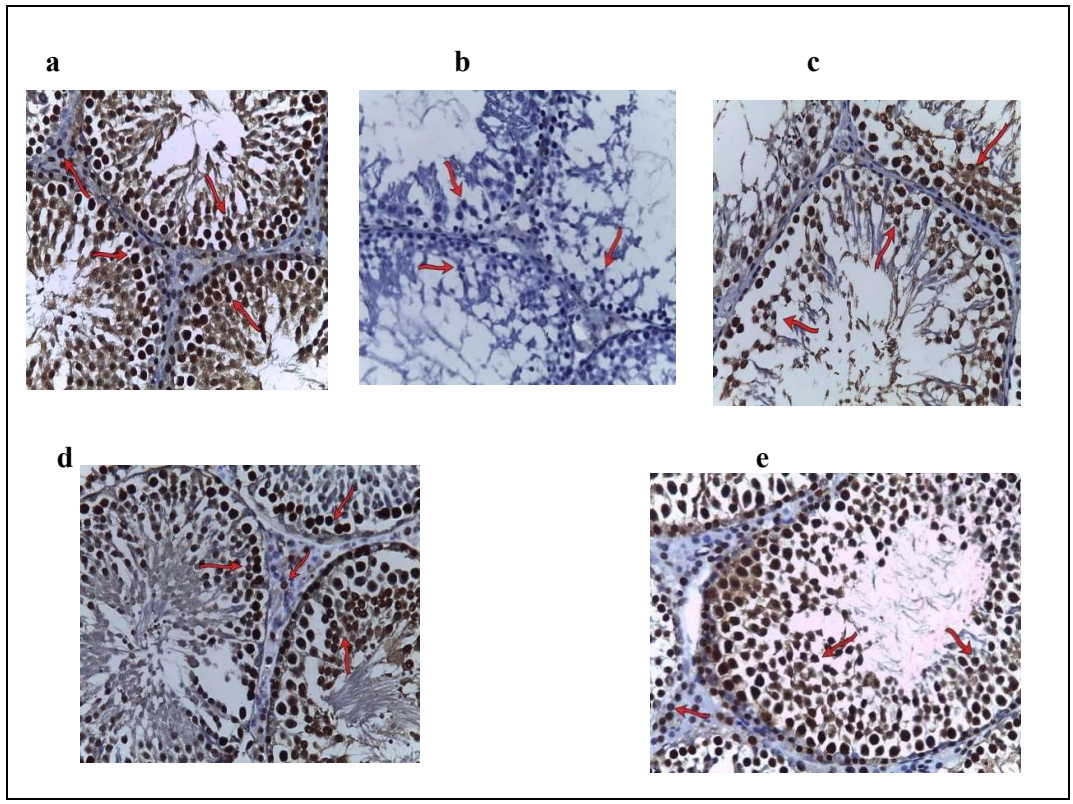


Figure 5: A Photomicrograph of a section in the testis of a rat from the **(control group)** shows +ve PCNA immune reactivity (red arrow). **(b) (cisplatin group)** shows negative immune reactivity of PCNA (**red arrow**). **(c) (melatonin group)** shows more expression of +ve PCNA immune reactivity (**red arrow**). **(d) (exosomes group)** shows increased +ve PCNA immune reactivity (**red arrow**). **(e) (melatonin and exosomes group)** shows near control immune expression of +ve PCNA_immune reactivity (**red arrow**). **(Anti PCNA, X400)**

Tumor necrosis factor-alpha (TNF- α) staining results:

Control group showed negative TNF- α immune reactivity, (figure 6a) while in cisplatin group showed strong TNF- α immune reactivity, (figure 6b). Melatonin, exosomes, and combined exosomes with melatonin groups showed decreased TNF- α immune reactivity, (figure 6c, 6d & 6e).

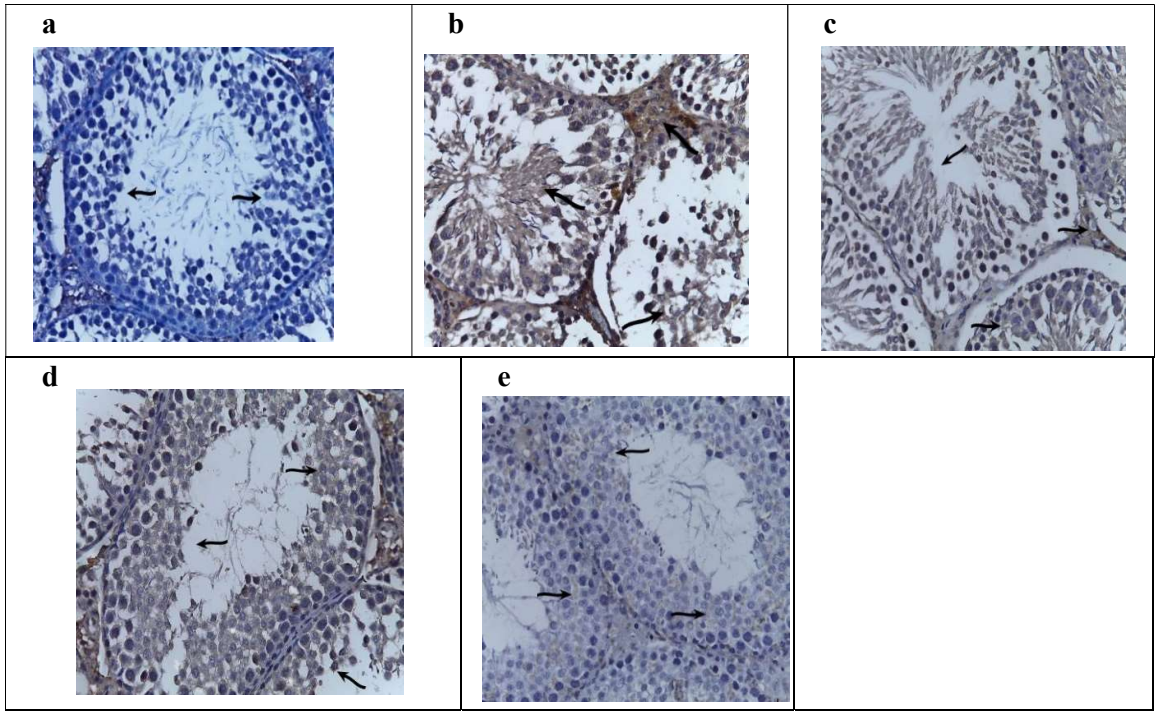


Figure 6: (a) A Photomicrograph of a section in the testis of a rat from the **(control group)** displays a -ve immunostaining of TNF- α (**black arrow**). **(b) (cisplatin group)** displays intensely +ve immunostaining response for TNF- α (**black arrow**). **(c) (**melatonin group) displays a moderate immunostaining reaction for TNF- α (**black arrow**). **(d) (exosomes group)** displays a slight immunostaining response for TNF- α (**black arrow**). **(e) (melatonin and exosomes group)** displays a weak immunostaining response for TNF- α (**black arrow**)

Morphometric and Statistical findings

All immunological parameter's mean area % and standard deviations of collagen fibers deposition, TNF- α, A.R and PCNA immuno-expression are featured in Tables from [1: 4] and in Histograms from [1: 4]. TNF- α and the average area % of collagen fiber accumulation both rose considerably (P-value < 0.01) in groups II while a decline in the mean area % of AR and PCNA immunostaining (P-value < 0.01) in groups II was noticed in comparison to group I. TNF- α immunostaining and the mean area % of collagen fiber accumulation both considerably declined (P-value < 0.01) while a more rise in the mean area % of AR and PCNA immunostaining (P-value < 0.01) than in group II was occurred in groups III, IV&V.

Table. [1]: The mean area percent and standard deviation of the collagen fibers accumulated in every group and comparing each group.
For all tables:
(a)=sig. with group (I) , (b)=sig. with group (II) , (c)=sig. with group (III) & (d)=sig. with group (IV) & (e)=sig. with group (V).

	control	**group 2**	**group 3**	**group 4**	**group 5**
Mean area %	0.14%	6.37%	2.60%	1.91%	1.21%
SD	0.01	3.24	1.16	0.81	0.91

Significance.at: P < 0.01	b,c,d,e	a,c,d,e	a,b,d,e	a,b,c,e	a,b,c,d

Table. [2]:AR immunostaining mean area percent & SD % across all groups.

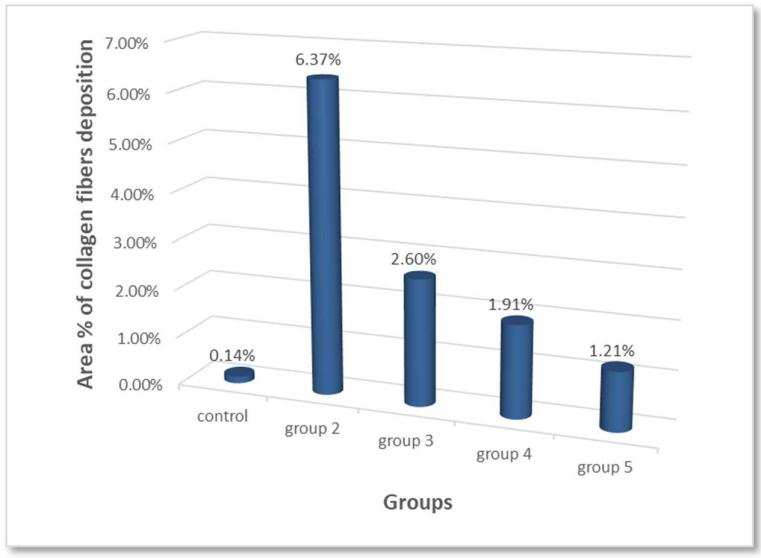
	control	**group 2**	**group 3**	**group 4**	**group 5**
Mean area %	19.18%	0.00%	10.26%	13.16%	16.00%
SD	9.59	0.00	6.11	6.94	7.26
Significance.at: P < 0.01	b,c,d,e	a,c,d,e	a,b,d,e	a,b,c,e	a,b,c,d

Table. [3]: PCNA immunostaining mean area percent & SD % across all groups.

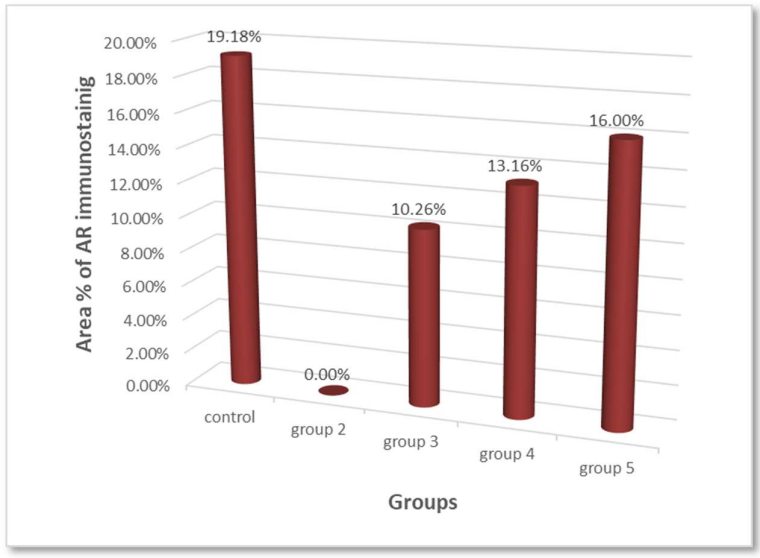
	control	**group 2**	**group 3**	**group 4**	**group 5**
Mean area %	7.39%	0.04%	2.90%	4.16%	5.92%
SD	4.05	0.00	1.85	3.15	3.86
Significance.at: P < 0.01	b,c,d,e	a,c,d,e	a,b,d,e	a,b,c,e	a,b,c,d

Table. [4]: TNF-α immunostaining mean area percent & SD % across all groups.

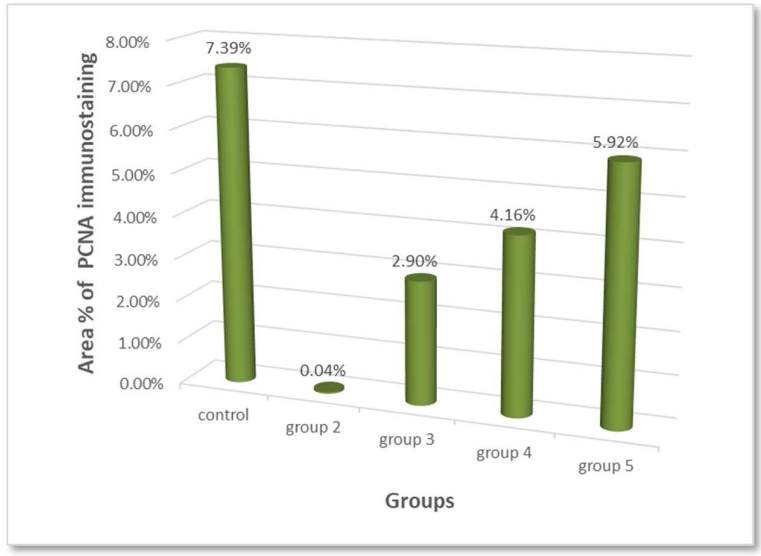
	control	**group 2**	**group 3**	**group 4**	**group 5**
Mean area %	0.00%	19.27%	13.70%	10.23%	5.08%
SD	0.00	13.09	8.55	6.87	3.62
Significance.at: P < 0.01	b,c,d,e	a,c,d,e	a,b,d,e	a,b,c,e	a,b,c,d



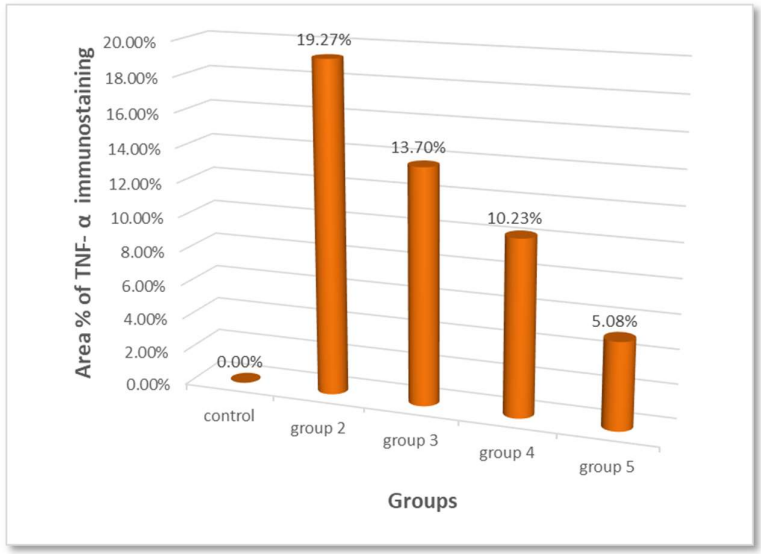
HISTOGRAM (1): Collagen fibers deposition % across all groups.



HISTOGRAM (2): AR immunostaining % across all groups.



HISTOGRAM (3): PCNA immunostaining % across all groups.



HISTOGRAM (4): average TNF-α immunostaining % across all groups.

DISCUSSION

The human testis is the most vulnerable organ to toxic agents attributed to its extraordinary cellular proliferation speed. Testicular impairment from cisplatin is the peak reported cases [13]. Cisplatin was administered in the present study as a single I.P. dose (8 mg/kg).

Group II (cisplatin group) in our study displayed vacuolations, BM detachment, distortion, and irregular outline of the majority of STs, decreased thickness of the germinal epithelium, relative widening of the interstitial spaces, and dilated congested interstitial blood vessels. When compared to controls, this group demonstrated a significant elevation in collagen fiber deposition, TNF- immunostaining, and a significant reduction in AR and PCNA immunostaining. These findings matched those of some researchers [14 - 17] and described by others [18, 19] who stated that, damage mediated by free radical is the primary mechanism of CIS-

provoked testicular toxicity as cisplatin reacts with water and oxidative stress overproduction (ROS) that foster cellular damage and apoptosis. Necrosis befell by lipid peroxidation in tissues, DNA destortion, and endoplasmic reticulum stress. It is also postulated by [20] that severe testicular mutilation- CIS initiated begin in Leydig cell malfunction (testosterone reduction).

Statistically increased Masson trichrome staining is explained by studies of [21&22] who suggested that cisplatin induced up-regulation of transforming growth factor beta (TGFβ) which is the passage marks of tissue fibrosis and profibrotic by ER stress and UPR signaling pathway. Our results showed a statistically increased TNF- α expression which is an indication of inflammation caused by cisplatin. In accordance with these results, previous studies of [23&24] showed that cisplatin triggers the inflammatory cytokines expression, comprising TNF-α and IL-1B, besides, cisplatin- provoked oxidative stress synchronized the expression of Nuclear factor kappa (NF-Kb), which sequentially increased TNF-α transcription.

Our results for AR and PCNA expression are analogous to the study of [25&26] which assessed that the decrease in the sum of AR, PCNA positive testicular germ cells after cisplatin treatment. Losing of spermatogenic cells followed the germ cells decrease and reduction in Leydig cells. Besides, the large vacuoles that appeared in testicular tubules were subsequently reported in the study of [27] which attributed it to Sertoli cells cytoplasmic processes retraction and extinction between different layers of spermatogenic cells that finally led to loose arrangement and easily separation of the cells.

Group III (melatonin group) in the present study exhibited a histological picture, comparable to cisplatin group, in the form of regular outline of the majority of the STs lined with the various phases of spermatogenic cells, vacuolations with areas of BM separation, wide interstitial tissue with congested blood vessels. This group showed a significant decrease ($P < 0.01$) in the mean area percent of Masson trichrome staining and TNF-α and a significant increase ($P < 0.01$) in the mean area percent of AR and PCNA as compared to group II. These findings are in line with earlier research cited by [7] that found that melatonin supplementation before and during cisplatin treatment may have a preventive role on testicular structure due to its antioxidant characteristics.

Some researchers[28, 29] explained that melatonin has both direct and indirect pathways as it can directly neutralize free radicals and related toxicants, so it protects antioxidative enzymes from oxidative damage. Every molecule of melatonin neutralizes two hydroxyl-free radicals (OH). Melatonin can also reduce the production of free radicals and electron leakage while boosting the effectiveness of mitochondrial electron transport. The study of [30] referred that the indirect pathway of melatonin depends on inhibition of the inducible nitric oxide synthase (iNOS) and NF-κB activation.

Exosomes convey their bioactive components, such as functional mRNAs, miRNAs, and lncRNAs, into recipient cells. They are the main therapeutic factors that promote and regulate MSCs' curative potential and paracrine function. Without the possible negative consequences of cell treatment, they can provide therapeutic results comparable to those of mesenchymal stem cells.[31].

Group IV (exosome group) of this study revealed a histological picture better than that of melatonin group as most of the STs have regular outline filled with germinal epithelium with relatively wide interstitial tissue. A substantial reduction ($P < 0.01$) in the mean area percent of Masson trichrome staining and TNF-α and a concidrable rise ($P < 0.01$) in the mean area percent of AR and PCNA as compared to group II. These results are consistent with [32- 34] who assumed that Through antioxidant, anti-inflammatory, and anti-apoptotic pathways, BMSC-exos protected against testicular toxicity; miRNAs enriched in exosomes, including as miR-155 and miR-146a, play a critical role in controlling inflammation. They were able to mediate repair

and regeneration by reduction of inflammatory transcription genes like IL-1β. They boosted the presence of proliferative PCNA+ cells and decreased inflammatory IL- β + and iNOS+ cells. Also, the study of [35] shown that miR-181c may regulate the expression of TNF-a, and that miR-181 overexpression increased the expression of the anti-inflammatory cytokine IL-10 considerably.

Some researchers[36, 37] explained that exosomal miRNAs have an anti-fibrotic effect by its ability to regulate reactive oxygen species, repairing damage of mitochondrial DNA and inflammatory activation. Also, exosomes can inhibit Fibroblast proliferation which is the key to fibrosis.

Group V (combined melatonin and exosomes) of the present study showed a histological picture, comparable to the control, with almost normal testicular architecture but some tubules showed a mild decrease in germinal lining. There was a significant decline ($P < 0.01$) in the mean area percent of Masson trichrome staining and TNF-α and a significant increase ($P < 0.01$) in the mean area percent of AR and PCNA compared to group II.in line with these results, the previous studies of [38, 39] have suggested that combined agents are superior to a single agent regimen. This is also in coincidence with other studies of [40, 41] which showed that combining exosomes with melatonin had an additional benefit in the treatment of acute lung diseases.

Also,some others [42,43] reported that either exosomes or melatonin treatment can result in increasing the expressions of proteins of anti-apoptotic and anti-oxidative markers but their combination results in further augmentation of these proteins. Melatonin, by its ability to act directly on damaged cells, can modify the molecular content of exosomes by interfacing with their trafficking intracellularly. So, combining melatonin with exosomes can result in great alteration in the biology of exosomes according to different signaling pathways in many diseases and pathological situations.

CONCLUSION

Each of melatonin and exosomes can protect against testicular toxicity induced by cisplatin, when given before and concurrently with cisplatin, with Exosomes exert more protection, however their combined administration can lead to superior outcomes.

Conflicts of interest:
No conflict of interest.

REFERENCE

1. **Rühle, A.; Perez, R. L.; Zou, B.; Grosu, A. L.;Peter E. Huber, P. E. and Nicolay,N. H. (2019):**The Therapeutic Potential of Mesenchymal Stromal Cells in the Treatment of Chemotherapy-Induced Tissue Damage. Stem Cell Reviews and Reports, 15(3):356-373.
2. **Nna, V. U.; Ujah, G. A.; Suleiman, J. B.; Mohamed, M.; Nwokocha, C.; Akpan, T. J.; Ekuma, H. C.; Fubara, V. V.; Kekung-Asu, C. B. and Osim, E. E. (2020):** Tert-butylhydroquinone preserve testicular steroidogenesis and spermatogenesis in cisplatin-intoxicated rats by targeting oxidative stress, inflammation and apoptosis. Toxicology, 441: 152528.
3. **Aldossary, S. A. (2019):** Review on pharmacology of cisplatin: Clinical use, toxicity and mechanism of resistance of cisplatin. Biomedical and Pharmacology journal, **12**:(1) :(7-15).
4. **Almeer, S. R. and Abdel Moneim, E. A. (2018):** Evaluation of the Protective Effect of Olive Leaf Extract on Cisplatin-Induced Testicular Damage in Rats. Oxidative Medicine and Cellular Longevity, 2018: (8487248):1-11.
5. **Shahanshah Khan, S.; Adhikari,J. S.; Rizvi, M. A. and Nabo Kumar Chaudhury, N. K. (2015):** Radioprotective potential of melatonin against 60Co γ-ray-induced testicular injury in male C57BL/6 mice 5. Journal of Biomedical Science, 22(61): 15 pages.

6. **Guo, X.B.; Zhai, J. W.; Xia, H.; Yang, J. K.; Zhou, J. H.; Guo, W. B.; Yang, C.; Xia, M.; Xue, K. Y.; Liu, C. D. and Zhou,Q. Z. (2021):** Protective effect of bone marrow mesenchymal stem cell-derived exosomes against the reproductive toxicity of cyclophosphamide is associated with the p38MAPK/ERK and AKT signaling pathways. Asian Journal of Andrology, 23, 386–391.
7. **Filobbos, S. A.; Amin, N. M. A.; Yacoub, M. F. Y. and Abd El-Hakim, K. R. (2020):** Possible Protective Effect of Melatonin on Cisplatin-Induced Testicular Toxicity in Adult Albino Rats. A Histological and Immunohistochemical Study. Egyptian Journal of Histology, 43: (3): 891-901.
8. **Kilic, u.; Kilic, E.; Tuzcu, Z.; Tuzcu, M.; Ozercan, H. B.; Yilmaz, O.; Sahin, F. & Sahin, K. (2013):** Melatonin suppresses cisplatin-induced nephrotoxicity via activation of Nrf 2/HO-1 pathway. Nutrition & Metabolism, 10(7): 1-8.
9. **Mohamady, R. and Mohamed, O. (2020):** Mesenchymal stem cells derived extracellular vesicles ameliorate cortical cerebellar changes-induced by aspartame in rats, Histological and immunohistochemical study. Egyptian Journal of Histology, 43(2), 380-389.
10. **El-Azab, N. E.; El-Mahalawaya, A. M.; Mostafa, O. and Sabry, D. (2018):** Histological and immunohistochemical study of the potential therapeutic impacts of bone marrow mesenchymal stem cells and exosomes for sciatic nerve crush injury model in rats. The Egyptian Journal of Histology, 41 (4):160-176.
11. **Bancroft, J. D. and Lyton. C. (2019):** The hematoxyline and eosin, Connective and other mesenchymal tissues with their stains and Immunohistochemical and Techniques. Eds. Suvarna, S. K.; Layton, C. and Bancroft, J. D. 8th ed. Ch.10& 12& 19. Pp. 126-138& 153-175& 337-394. Churchill Livingstone of El Sevier, Philadelphia.
12. **Seys, L. J.; Verhamme, F. M.; Schinwald, A.; Hammad, H.; Cunoosamy, D.M.; Bantsimba, M. C, et al. (2015): Role of B cell–activating factor in chronic** obstructive pulmonary disease. American journal of respiratory and critical care medicine, 192(6):706.
13. **Eida, H. A.; Abdelkaderb, F. N.; Abd El-Raoufa, M. O.; M. H.; El-Sayehb, M. B. & El-Densharyb,S. E.(2016):** Protective effect of L- carnitine against cisplatin induced testicular toxicity in rats. Al-Azhar.J. Pharm Sci. 53: (1): 123-142.
14. **Köroğlu, K. M.; Çevik, Ö; Şener, G. and Ercan, F. (2019):** Apocynin alleviates cisplatin-induced testicular cytotoxicity by regulating oxidative stress and apoptosis in rats. Andrologia, 51: e13227.
15. **Azarbarz, N.; Seifabadi, Z. S.; Moaiedi, M. Z. and Mansouri, E. (2020):** Assessment of the effect of sodium hydrogen sulfide (hydrogen sulfide donor) on cisplatin-induced testicular toxicity in rats. Environmental Science and Pollution Research, 27:8119–8128.
16. **Mesbahzadeh, B.; Taheri, M. H.; Aliparast, M. S.; Baniasadi, P. and Hosseini, M. (2021):** The protective effect of crocin on cisplatin-induced testicular impairment in rats. B.M.C. Urol., 21:(117): 1-9.
17. **Hassen, M.T.; Mohamed, H. K.; Montaser , M. M.; El Sharnouby, M. E.; Awad, N. and Ebiya, R. A. (2021):** Molecular, Immunomodulatory, and Histopathological Role of Mesenchymal Stem Cells and Beetroot Extract on Cisplatin Induced Testicular Damage in Albino Rats. Animals (Basel), 11(4):1142.
18. **Yadav, C. Y. (2019):** Effect of cisplatin on pancreas and testies in Wistar rats: biochemical parameters and histology. Heliyon, 5: e02247.
19. **Hamza, A. A.; Elwy, H. M. and Badawi, A. M. (2016):** Fenugreek seed extract attenuates cisplatin-induced testicular damage in Wistar rats. Andrologia, 48(2): 211–221.
20. **Wang, X.;Gu, H.; Qin, D.; Yang, L.; Huang, W.; Essandoh, K.;Wang, Y.; Caldwell, C.C.; Peng, T.; Zingarelli, B. andFan, G.C. (2015):** ExosomalmiR-223 contributes tomesenchymal stem cell-elicited Cardioprotection Polymicrobial sepsis. Sci. Rep., 5: 13721.

21. **Lenna, S. and Trojanowska, M. (2012):** The role of endoplasmic reticulum stress and the unfolded protein response in fibrosis. Curr. Opin. Rheumatol., 24:663–668.
22. **Yucel,C.; Arslan, F. D.; Ekmekci, S.; Ulker,V.; Kisa, E.; Yucel, E. E.; Ucar, M.; Ilbey, Y. O; Celik, O; Basok, B. I. and Kozacioglu, Z. (2019):** Protective Effect of All-Trans Retinoic Acid in Cisplatin-Induced Testicular Damage in Rats.World J. Mens Health, 37(2): 249–256.
23. **Sherif, I. O.; Abdel-Aziz, A.; Sarhan, O. M. (2014):** Cisplatin-induced testicular toxicity in rats: the protective effect of arjunolic acid. J. Biochem. Mol. Toxicol., 28(11): 515-521.
24. **Fouad, A. A.; Qutub, H. O.; Fouad, A. E. A. Audeh, A. M. and Yogesh Chand Yadav, Y. C. (2016):** Effect of Ficus benghalensis L. Latex extract (FBLE) on cisplatin induced hypotension and renal impairment in Wistar rats. Biochem. Pharmacol., 5:216.
25. **Afsar, T.; Razak, S.; khan, M. R. and Almajwal, A. (2017):** Acacia hydaspica ethyl acetate extract protects against cisplatin-induced DNA damage, oxidative stress and testicular injuries in adult male rats. BMC Cancer, 17(1): 883.
26. **Kanter, M.; Aktas, C.; Erboga, M. (2013):** Curcumin attenuates testicular damage, apoptotic germ cell death, and oxidative stress in streptozotocin-induced diabetic rats. Mol. Nutr. Food Res., 57, 1578–1585
27. **Zhao, Y. M.; Gao, L. P.; Zhang, H. L. Guo, J. X. and Guo, P. P. (2014):** Grape seed proanthocyanidin extract prevents DDP induced testicular toxicity in rats. Food & Function, 5(3): 605–611.
28. **Sun, K. CH; Chen, H. CH; Chang, L. CH; Chiang, J. H.; Sung, P. H.; Chen, K. H.; Chen, Y. L.; Chen, S. Y.; Kao, G. S.; Chang, H. W.; Lee, M. S. and Yip, H. K. (2017):** Melatonin treatment enhances therapeutic effects of exosomes against acute liver ischemia-reperfusion injury. Am. J. Transl.Res., 9:(4): 1543-1560.
29. **Aminjan H. H.; Abtahi S. R.; Hazrati E.; Chamanara M.; Jalili M. and Paknejad B. (2019):** Targeting of oxidative stress and inflammation through ROS/NF-kappaB pathway in phosphine-induced hepatotoxicity mitigation. Life Sci., 232:116607.
30. **Asghari, M. H.; Moloudizargari, M.; Baeeri, M.; Baghaei, A.; Rahimifard, M.; Solgi, R.;Jafari, A.; Haghi Aminjan, H.; Hassani, S.; Moghadamnia, A.A.; Ostad, S. N. and Abdollahi, M. (2017):** On the mechanisms of melatonin in protection of aluminum phosphide cardiotoxicity. Arch. Toxicol., 91: 3109–3120.
31. **Zhang, Y.; Bi, J.; Huang, J.; Tang, Y.; Du, S. and Li, P. (2020):** Exosome: A Review of Its Classification, Isolation Techniques, Storage, Diagnostic and Targeted Therapy Applications. Int. J. Nanomedicine, 15:6917-6934.
32. **Park,D. J.; Park, J. E.; Lee, S. H.; Eliceiri, B. P.; Choi, J. S.and Seo, Y. J. (2021):** Protective effect of MSC-derived exosomes against cisplatin-induced apoptosis via heat shock protein 70 in auditory explant model. Nanomedicine: Nanotechnology, Biology, and Medicine, 37:102447:12 Pages.
33. **Pirisinu, M. (2023):** The long journey of extracellular vesicles towards global scientific acclamation. Advanced Pharmaceutical Bulletin, 13:(2):1-20.
34. **Fang, W. and Vangsness, T. (2020):** Implications of Anti-Inflammatory Nature of Exosomes in Knee Arthritis. Cartilage, 13(2):204-212.
35. **Hutchison, E.R.; Kawamoto1, E. M.; Taub, D. D.; Lal, A.; Abdelmohsen, K.; Zhang,Y.; Wood, W. H.; Lehrmann, E.; Camandola, S.; Becker, K. G.; Gorospe, M. and Mattson, M. P. (2013):** Involvement of miR-181 in Neuroinflammatory Responses of Astrocytes. Glia, 61(7): 1018–1028.
36. **Jia, Y. C.; Ding, Y. X.; Mei, W. T.; Wang, Y. T.; Zheng, Z.; Qu, Y. X, et al. (2021):** Extracellular vesicles and pancreatitis: mechanisms, status, and perspectives. International journal of biological sciences, 17(2): 549–561.
37. **Huang, Y. and Yang, L. (2021):** Mesenchymal stem cell-derived extracellular vesicles in therapy against fibrotic diseases. Stem Cell Res &Ther., 12(1): 435-447.

38. **Zaouali, M.A.; Boncompagni E; Reiter, R. J.; Bejaoui, M.; Freitas, I.; Pantazi, E.; Folch-Puy, E.; Abdennebi, H. B.; Garcia-Gil, F.A. and Rosello-Catafau ,J. (2013):** AMPK involvement in endoplasmic reticulum stress and autophagy modulation after fatty liver graft preservation: a role for melatonin and trimetazidine cocktail. J. Pineal. Res., 55:65-78.
39. **Willars, C. (2014):** Update in intensive care medicine: acute liver failure. Initial management, supportive treatment and who to transplant. Curr. Opin. Crit. Care, 20: 202-209.
40. **Yip, H.K.; Chang, Y.C.; Wallace, C.G.; Chang, L.T.; Tsai, T.H.; Chen, Y.L.; Chang, H.W.; Leu, S.; Zhen, Y.Y.; Tsai,C.Y.; Yeh, K.H.; Sun, C.K. and Yen, C.H. (2013):** Melatonin treatment improves adipose-derived mesenchymal stem cell therapy for acute lung ischemia-reperfusion injury.J .Pineal. Res., 54: 207-221.
41. **Sun, C.K.; Lee, F.Y.; Kao, Y.H.; Chiang, H.J.; Sung, P.H.; Tsai, T.H.; Lin, Y.C.; Leu, S.; Wu, Y.C.; Lu, H.I.; Chen, Y.L.; Chung, S.Y.; Su, H.L. and Yip, H.K. (2015):** Systemic combined melatonin-mitochondria treatment improves acute respiratory distress syndrome in the rat. J. Pineal. Res., 58: 137-150.
42. **Wang, T. E; Lai, Y. H;Yang, K. C.; Lin, S.J; Chen, C. L. and Tsai, P. S. (2020):** Counteracting Cisplatin-Induced Testicular Damages by Natural Polyphenol Constituent Honokiol. Antioxidants, 9:(8): 23Pages.
43. **Novais, A.A.; Chuffa, L.G.D; Zuccari, D.A.P. and Reiter, R.J. (2021):** Exosomes and Melatonin: Where Their Destinies Intersect.Frontiers in Immunology, 12(692022): 1-11.