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Pinealectomy in Ehrlich ascites-inoculated mice increased the risk of liver and kidney injury

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Abstract: Background: Pineal gland impairment, characterized by disrupted melatonin synthesis, which collectively impairs liver and kidney homeostasis. This study explored the role of pineal impairment (Pnx) in mice inoculated with Ehrlich ascites cells in increasing susceptibility to liver and kidney toxicity.

Methods and results: Female Swiss albino mice were divided into three groups (of five animals each) that were engrafted with Ehrlich tumor (ET) cells; one served as a control, one as a sham, and the last as a Pnx group. Alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activities were significantly elevated, whereas total protein and albumin levels were markedly decreased in Pnx mice. In addition to the elevation of creatinine, urea, and uric acid levels, which were also seen in the Pnx group. Antioxidants SOD, CAT, GSH, GPx were significantly decreased, and an increase in GST in liver and kidney tissues with a marked increase in oxidative stress marker MDA. Histopathological investigations reflected changes in liver and kidney architecture with vascular congestion, apoptosis, and atrophy in Pnx mice.

Conclusions: Taken together, our findings indicated that depleting melatonin in Ehrlich ascites-inoculated mice increased the risk of hepatotoxicity and kidney toxicity.

keywords: Ehrlich, pinealectomy, oxidative stress, antioxidants, hepatotoxic

1. Introduction

Cancer is expected to rise by 70% globally by 2030, with rising incidence, hospitalization, and death rates **[1].** Ehrlich carcinoma, a spontaneous mammary adenocarcinoma in mice, has been extensively studied as an in vivo tumor xenograft model **[2, 3]**. Ehrlich tumor is the most common model of breast cancers **[4].** Ehrlich tumor cells are highly proliferating, undifferentiated, and chemotherapy-sensitive. Ehrlich tumor cells can be xenografted to create solid tumors or utilized as ascites. Solid Ehrlich tumor were similar to human breast tumors in that they showed elevated expression of immune factors **[5].** Pineal gland is a photo neuroendocrine organ in the midline of the brain, and the gland is a part of the epithalamus, which is located between the diencephalon and mesencephalon and connected to the third ventricle by its recesses **[6, 7].** The gland produces and secretes serotonin, dimethyltryptamine, and melatonin**[8].**

Melatonin (N-acetyl-5-methoxytryptamine), is an indole amine and neuro-hormone synthesized and secreted primarily by the pineal gland, but it can also come from a different other places, including the gastrointestinal tract, adrenal gland, genital glands, placenta, uterus, bone marrow, skin, cerebellum, retinal photoreceptors, lens, thymus, heart, platelets, eosinophilic leukocytes, natural killer cells, mast cells, T- lymphocytes, and other organs **[9, 10].** Melatonin-dependent light regulates the circadian cycle**[11].** Levels of melatonin synthesis and secretion increase at night and start to decrease in the morning and during the day **[12].** Numerous social situations, such as working at night, working shifts, and being around artificial light at night, might interfere with circadian rhythms by reducing melatonin synthesis and secretion **[13].** Elevated melatonin levels at night promote optimal homeostatic metabolic cycles, which shield the

body from the onset of many diseases, including cancer, sleep disruptions, aging, metabolic processes, heart disease, diabetes, mental problems, and obesity **[14, 15].** Antioxidant is described as a substance that directly scavenges ROS or indirectly acts to upregulate antioxidant defences or inhibit ROS production, and protect tissues against oxidative stress**[16].** The researchers showed that melatonin is associated with immune modulation, oxidative stress, hematopoiesis, and several anticancer mechanisms, including apoptosis induction, cell proliferation inhibition, tumor growth and metastasis reduction, drug resistance reduction in cancer treatment, and amplifying the therapeutic effects of traditional anticancer therapies **[17].**

This study aimed to assess the impact of pineal impairment in Ehrlich solid tumor-inoculated mice on boosting hepatotoxicity and nephrotoxicity.

2. Materials and methods

2.1. Animals

Female SWISS mice weighing 25g ± 3g were provided by the Egyptian Vaccine Company (VACSERA, Cairo, Egypt). Mice were allowed to adjust to the circumstances for one week in the animal facility of Zoology Department, Faculty of Science, and Mansoura University. Mice were housed at a constant temperature (20°C ± 5°C) and provided standard mouse chow and water supplements, and with 12-h light/12-h dark cycles.The current study was approved by Mansoura University's Institutional Ethics Committee approval number: (Sci-Z-M-2022-81).

2.2. Light source

The animal house was provided with white ceiling fluorescent tubes, and animal cages were positioned to ensure that the light intensity average was (190-210) lx. Light intensity was verified with a light meter (Light meter UNI-T UT383S, China), and during the experimental period, 21 days, mice were maintained at 24-h light.

2.3. Ehrlich Ascites Cells

Female mice with Ehrlich Ascites cells (EACs) at the peritoneal cavity were supplied by the National Cancer Institute (NCI, Cairo, Egypt), and then cells were withdrawn at the beginning of the experiment to be injected subcutaneously into the back of female SWISS mice.

2.4. Surgical procedures for Pinealectomy

Following acclimation, female Swiss mice were fasted for 12 hours and then were anesthetized with 15 mg/kg xylazine and 30 mg/kg ketamine**[18]**. They underwent a longitudinal scalp incision to expose the lambda suture. The skull around the lambda suture was gently removed, and the pineal gland was extracted using fine forceps. The skull bone was returned to its normal position, and the scalp was sutured. The surgery was completed in 30 minutes**.** Following surgery, the animals were given amoxicillin and ketoprofen (single dose—intramuscular)**[19]**. All operated mice were given two weeks to recover before starting the experiment.

2.5. Experimental design and mice treatment

Following acclimatization for one week, female Swiss mice were randomly divided into 3 groups with 5 mice per group, with 24-h light exposure (light was emitted by light meter with light intensity average (190 – 210) lx for 21 days, as follows:

Group 1 (Control group): Mice of this group were injected subcutaneously at the back area with (2.5×10^6EACs) on the first day of the experiment.

Group 2 (Sham group): Before starting the experiment, mice of this group were prepared for opening in the skull, as in pinealectomy, but without removal of the gland, and after 2 weeks of recovery, they were injected subcutaneously with (2.5×10^6 EACs).

Group 3 (Pnx group): Before starting the experiment, mice of this group underwent pinealectomy**[19],** and after 2 weeks of recovery, they were injected subcutaneously at the back with (2.5×10^6 EACs).

2.6. Samples collection

At the end of the experimental period (21 days), mice from all groups were anesthetized with ketamine/xylazine (0.1 ml/100 g, ip)**[20]** then blood samples were collected from the heart by cardiac puncture and then animals were euthanized to obtain kidneys and livers. Blood was centrifuged at 1500 rpm for 20 mins. Sera were stored at -20°C for biochemical assays.

Part of the liver and kidney was homogenized in ice-cold PBS, then homogenates were centrifuged at 5000 rpm for 20 minutes and stored at 20°C for biochemical analysis. Other parts of the liver and kidney were fixed in a 10% neutral formalin solution for 48 hours for histopathological investigations.

2.7. Biochemical analysis

Liver functions such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities, total protein, and albumin contents were determined by colorimetry in serum using kits purchased from the Biodiagnostic Company, Dokki, Giza, Egypt**[21].**

Kidney functions such as creatinine, urea, and uric acid activities were determined colorimetry in serum using kits purchased from the Biodiagnostic Company, Dokki, Giza, Egypt**[22].**

Antioxidants and oxidative stress markers such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx), glutathione-s-transferase (GST), and malondialdehyde (MDA) were measured colorimetrically in liver and kidney homogenate by using kits purchased from the Biodiagnostic Company, Dokki, Giza, Egypt**[21].**

2.8. Histopathology study

Liver and kidney tissues were fixed, dehydrated, cleared, and immersed in paraffin, then sectioned (5 µm). Sections were later stained with hematoxylin and eosin. Stained sections were examined under an Olympus light microscope, and images were captured using an Amscope MU1000 camera.

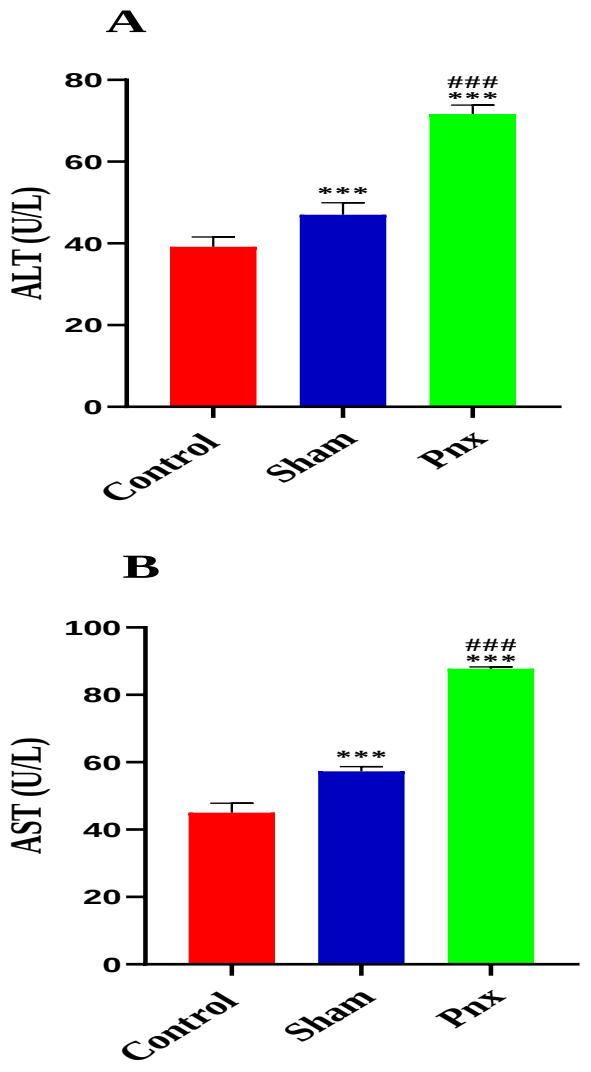
2.9. Statistical analysis

GraphPad Prism 8.0 software was used for statistical data analysis. The results of the experiments were expressed as the mean ± standard deviation (SD). One-way ANOVA and post hoc (Tukey and Dunnett) tests for multiple comparisons were performed.

3. Results and Discussion

3.1. Serum levels of liver function parameters

Across the experimental groups **(figure 1)**, showed significant changes in liver function parameters, including the activity of ALT, AST, and ALP, and the concentration of total protein and albumin. Pnx group exerted a significant ($P < 0.001$) increase in the serum levels of liver function enzymes, including ALT, AST, and ALP, with a significant ($P < 0.001$) decrease in the serum levels of albumin and total protein compared with those in the control and sham groups. Furthermore, the results showed a significant ($P < 0.001$) increase in the levels of ALT, AST, and ALP in addition to a significant ($P < 0.001$) decrease in the serum levels of albumin and total protein contents in the sham mice compared with the control mice.



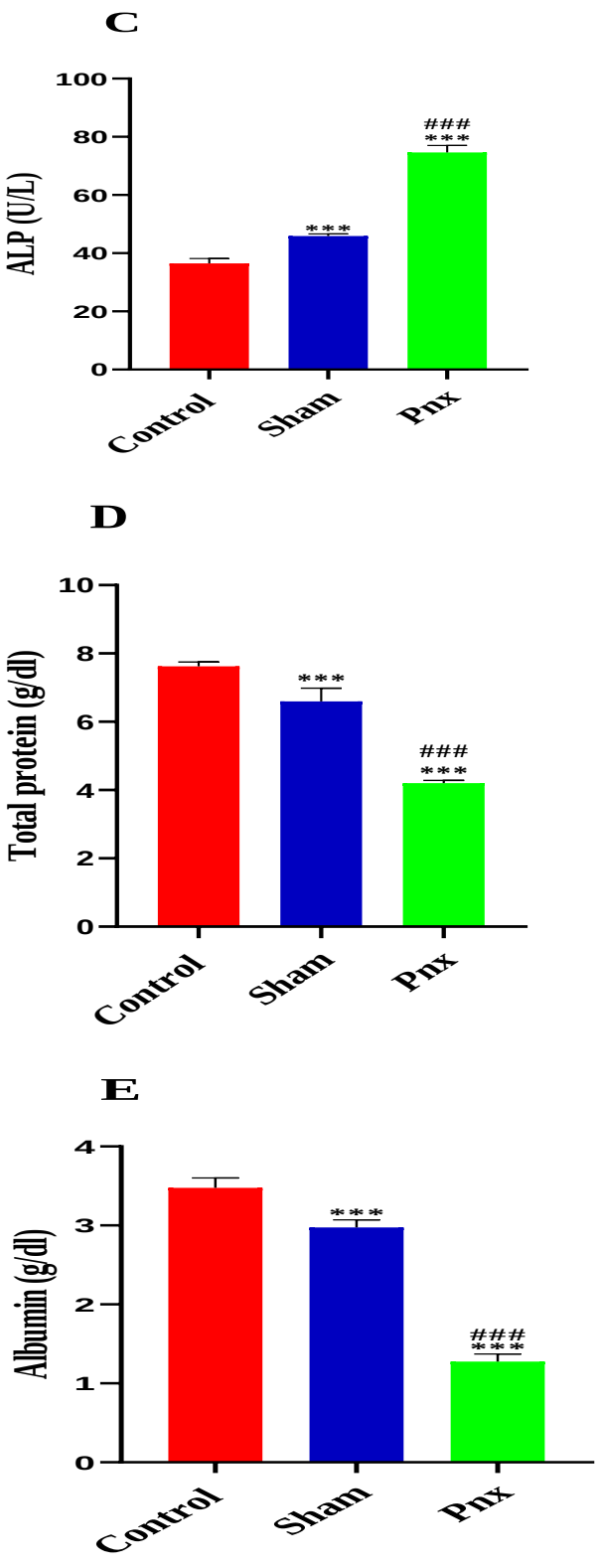


Figure 1: Serum Levels of alanine aminotransferase (ALT) (U/L) **A**, aspartate aminotransferase (AST) (U/L) **B**, alkaline phosphatase (ALP) (U/L) **C**, total protein (g/dl) **D**, and albumin (g/dl) **E** of mice in control and other groups. Data were shown as mean ± SD (*n=4*). ***, ### significant at *P < 0.001.* *** Comparison versus the control groups. ### Comparison versus the sham group.

3.2. Serum levels of kidney function parameters

Changes in kidney function parameters, including the creatinine, urea, and uric acid content in all study groups, were assessed in **(Figure 2)**. The data showed that the removal of the pineal gland in Pnx mice caused a significant (P < 0.001) increase in serum levels of kidney function parameters compared with those in the control and sham mice. Otherwise, the sham group showed a significant (P < 0.001) increase in the serum levels of kidney function parameters compared with the control group.

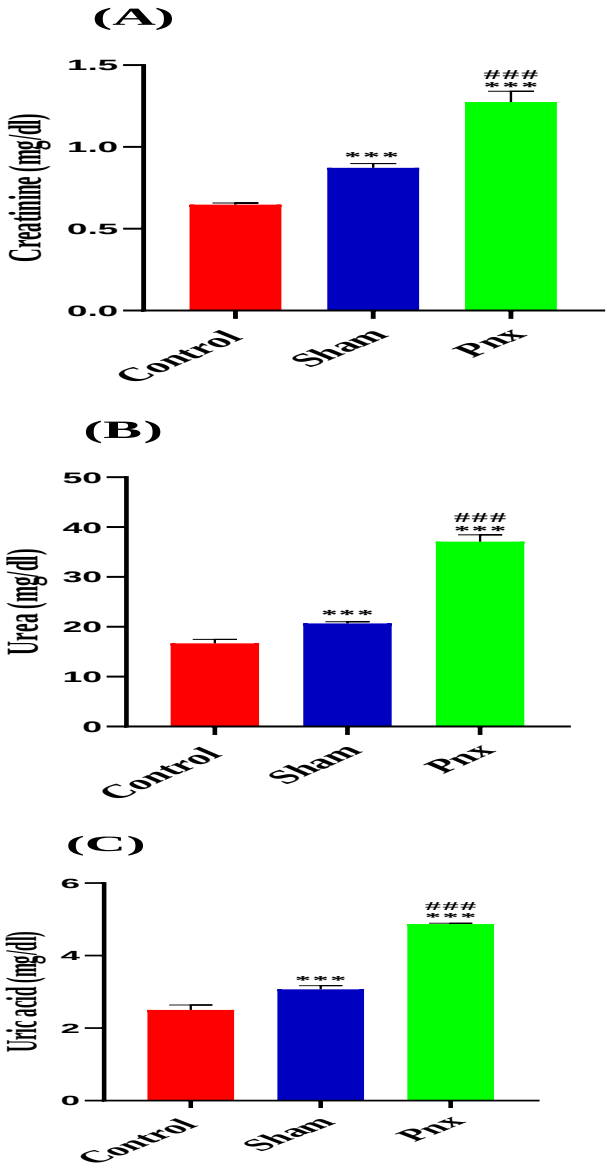


Figure 2: Serum Levels of creatinine (mg/dl) (A), urea (mg/dl) (B), uric acid (mg/dl) (C) of mice in control and other groups. Data were shown as mean ± SD (n=4). ***, ### significant at P < 0.001. *** Comparison versus

the control groups. ### Comparison versus the sham group.

3.3. Antioxidants and oxidative stress marker levels in the liver and kidney

Pnx mice showed a significant ($P < 0.001$) decrease in liver and kidney antioxidant enzyme activity including SOD, CAT, GPx, and GSH content with a significant ($P < 0.001$) increase in the activity of GST, and the oxidative stress marker (MDA) content compared with those in the control and sham mice. For instance, liver antioxidant enzyme activity of sham mice was significantly ($P < 0.001$) lower than that of control and sham mice. On the other hand, GST activity and the oxidative stress marker (MDA) content were significantly ($P < 0.001$) higher than those in the control mice **(Figure 3 and 4) .**

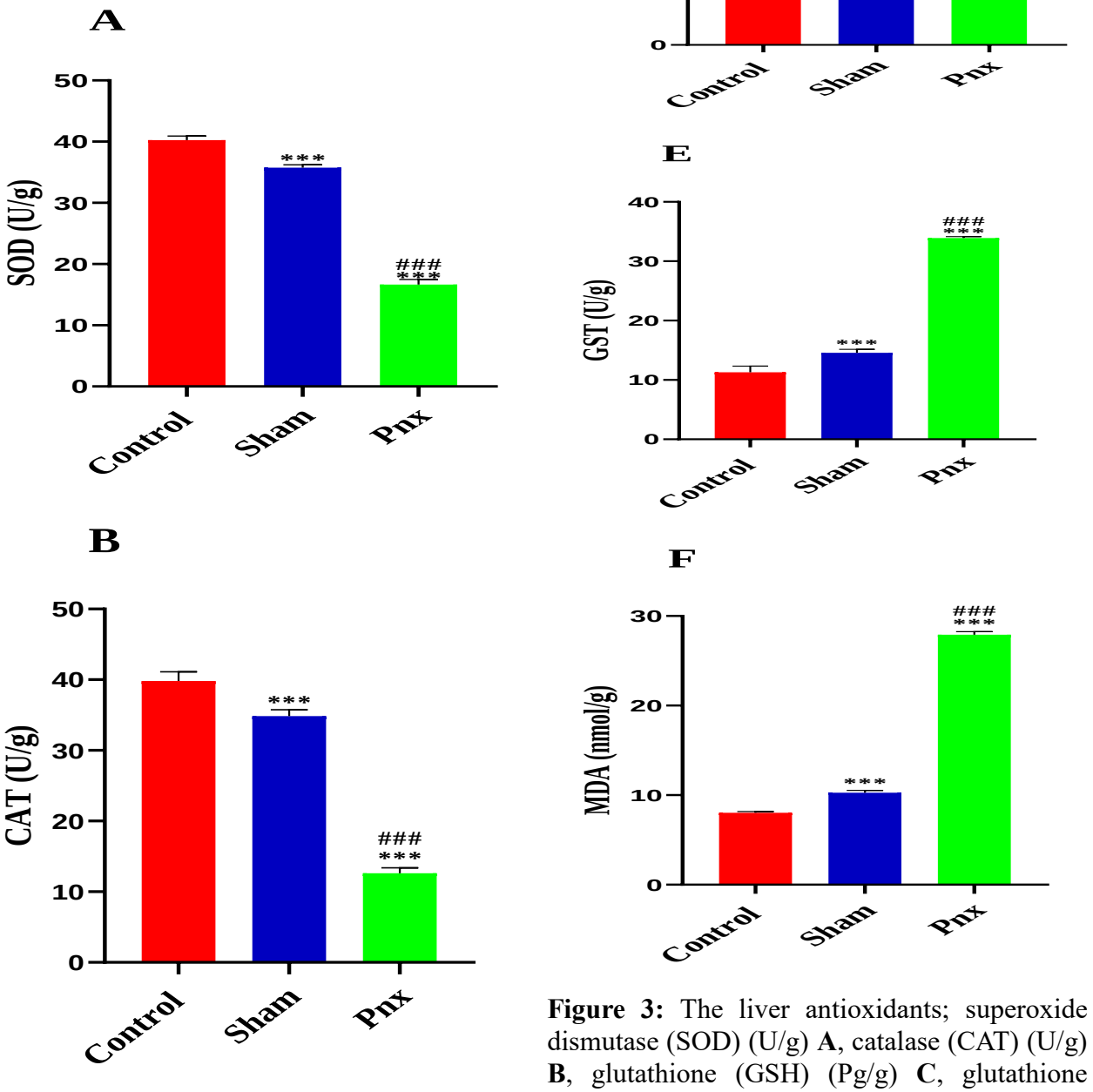
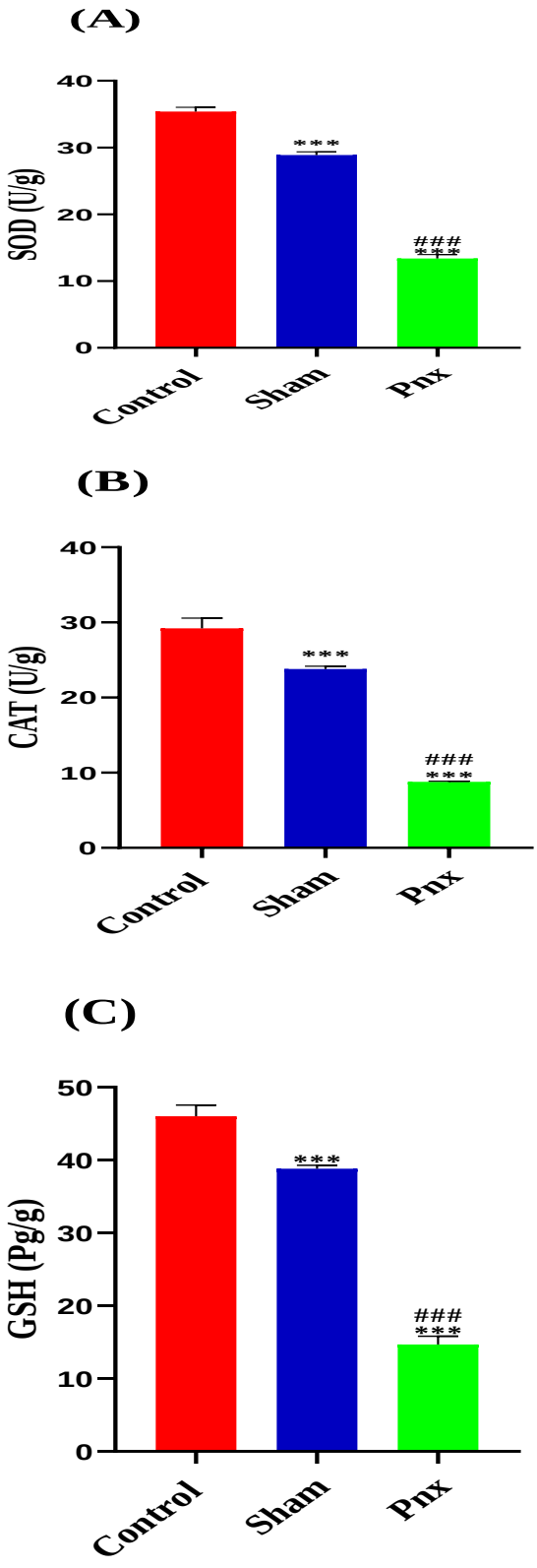


Figure 3: The liver antioxidants; superoxide dismutase (SOD) (U/g) **A**, catalase (CAT) (U/g) **B**, glutathione (GSH) (Pg/g) **C**, glutathione

peroxidase (GPx) (U/g) **D**, glutathione-s-transferase (GST) (U/g) **E**. The liver Oxidative stress marker; malondialdehyde (MDA) (nmol/g) **F** of mice in control and other groups. Data were shown as mean ± SD (*n=4*). ***, ### significant at *P < 0.001*. *** Comparison versus the control groups. ### Comparison versus the sham-group.



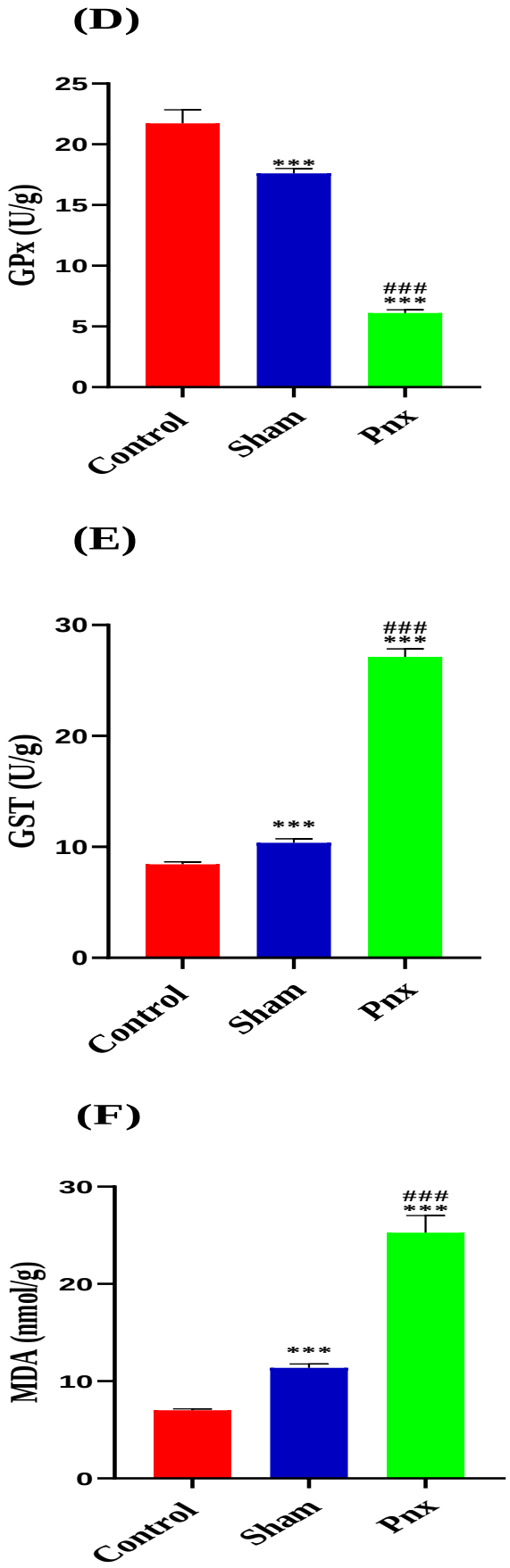


Figure 4: The kidney antioxidants; superoxide dismutase (SOD) (U/g) **(A)**, catalase (CAT) (U/g) **(B)**, glutathione (GSH) (Pg/g) **(C)**, glutathione peroxidase (GPx) (U/g) **(D)**,

glutathione-s-transferase (GST) (U/g) **(E)**. The kidney Oxidative stress marker; malondialdehyde (MDA) (nmol/g) **(F)** of mice in control and other groups. Data were shown as mean ± SD (*n=4*). ***, ### significant at $P < 0.001$. *** Comparison versus the control groups. ### Comparison versus the sham group.

3.4. Histopathological alterations in the liver and kidney

Histopathological examination of liver tissues from **the control** mice revealed mild vascular congestion, portal round cells infiltration, biliary proliferative reaction, beside micro and macro-hepatocellular steatosis **(Figure 5)**. Liver section from the sham group showed moderate congestion and fibroplasia of portal blood vessels, portal round cells aggregation, mild biliary reaction, frequent sinusoidal dilatation and Kupffer cells hypertrophy and hyperplasia were also recorded as well **(Figure 6).** Examined sections from liver of the Pnx group, showed portal vascular dilatation, biliary proliferation, peri-portal round cells aggregation, lymphatic and lympho-vascular dilatation in addition to, focal portal fibroplasia and many hepatocellular apoptosis **(Figure 7)**. Kidney tissues from the control mice revealed mild to moderate tubular epithelial degeneration and renal vascular congestion, lymphocytic aggregation and glomerular lobulation **(Figure 8)**. Section from the sham group showed moderate tubular epithelial degeneration, renal vascular congestion, and interstitial peri-vascular and lymphocytic aggregation; with occasional intra-tubular hyaline cast formation glomerular lobulation **(Figure 9)**. Examined sections from kidney of the **Pnx** group, showed marked perivascular aggregation of lymphocytes, vascular congestion, glomerular lobulation, and tubular epithelial atrophy as well **(Figure 10)**.

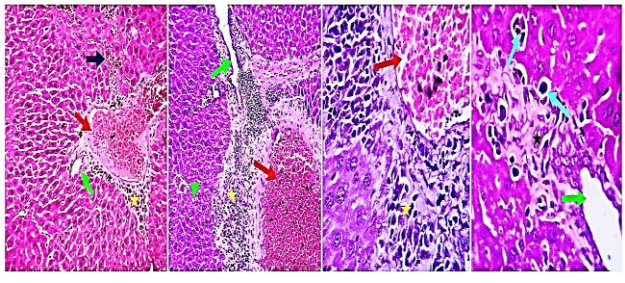


Figure 5: Photomicrographs of hematoxylin and eosin-stained hepatic tissue of control group revealing mild vascular congestion (red arrow), portal leukocytic infiltration (yellow asterisk), biliary proliferative reaction (green arrows), and fat degeneration, beside micro and macro-hepatocellular steatosis (brown arrow). H&E, × 200&400 respectively.

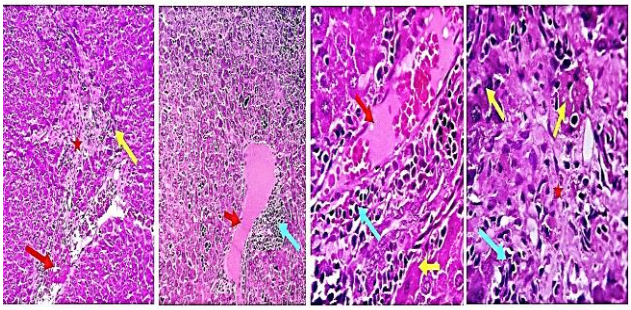


Figure 6: Photomicrographs of hematoxylin and eosin-stained hepatic tissue of the sham group showing moderate congestion of portal blood vessels (red arrow), leukocytic infiltration (yellow asterisk), mild biliary reaction (green arrow), sinusoidal dilatation (blue arrow), and portal fibroplasia (black asterisk). H&E, × 200&400 respectively.

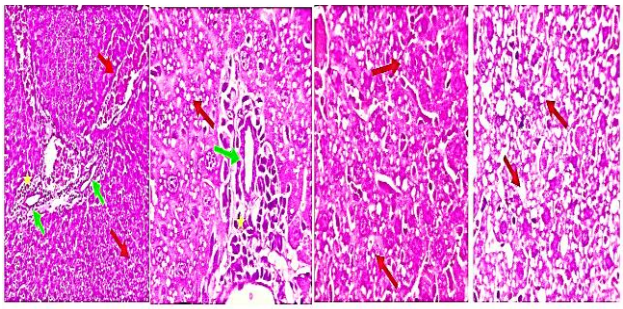


Figure 7: Photomicrographs of hematoxylin and eosin-stained hepatic tissue of the Pnx group showing portal vascular dilatation and congestion (red arrow), fibroplasia (red asterisk), abundant of apoptotic cells (yellow arrow), and periportal round cells aggregation (blue arrow). H&E, × 200&400 respectively.

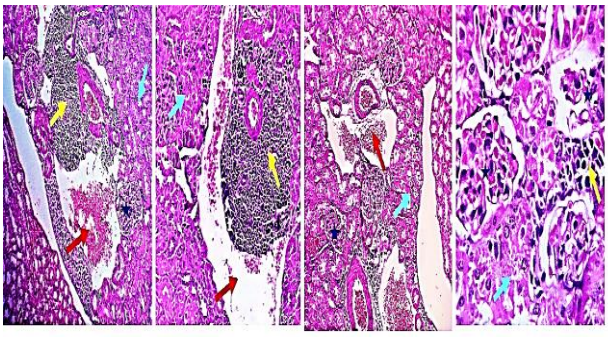


Figure 8: Photomicrographs of hematoxylin and eosin-stained kidney sections of the control group showing mild to moderate renal vascular

congestion (red arrow), perivascular lymphocytic aggregation (yellow arrow), glomerular lobulation (asterisk) and mild tubular epithelial degeneration (blue arrow). H&E, × 100&400 respectively.

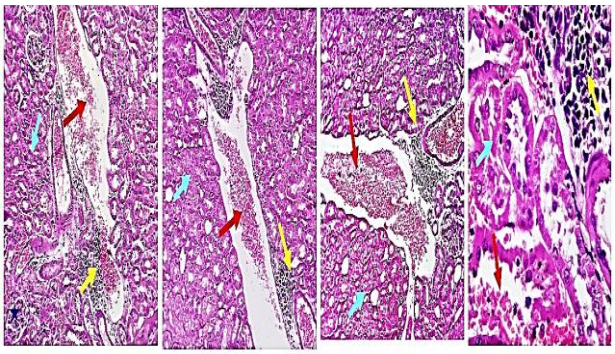


Figure 9: Photomicrographs of hematoxylin and eosin-stained kidney sections of the sham group showing moderate renal vascular congestion (red arrow), interstitial peri-vascular and lymphocytic aggregation (yellow arrow), tubular epithelial degeneration (blue arrow) with occasional intra-tubular hyaline cast formation glomerular lobulation. H&E, × 100&400 respectively.

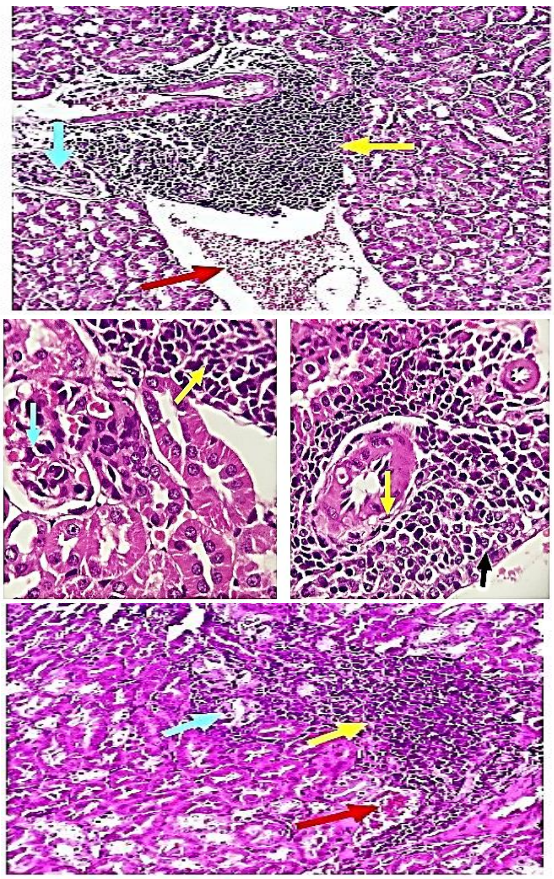


Figure 10: Photomicrographs of hematoxylin and eosin-stained kidney section of the Pnx group revealing marked perivascular aggregation of lymphocytes (yellow arrow), vascular congestion (red arrow), glomerular lobulation (blue arrow), and tubular epithelial atrophy (black arrow). H&E, × 100&400 respectively.

Discussion

The current study sought to determine the effect of pineal impairment in Ehrlich solid tumor-inoculated mice on increased hepatotoxicity and nephrotoxicity. The liver is primarily a metabolic organ with multiple functions that include toxin detoxification and metabolic activity regulation **[23].** The kidney performs several crucial homeostatic functions. Among them are waste removal (NH3), fluid/electrolyte balance, metabolic blood acid-base balance, the synthesis of hormones for blood pressure, calcium/potassium homeostasis, and red blood cell production**[24].** Our findings revealed that pinealectomy enhances the Ehrlich solid tumor-induced hepatotoxic and nephrotoxic consequences, which are characterized by increased oxidative stress. Oxidative stress is characterized as "an imbalance between oxidants and antioxidants in favor of the oxidants, resulting in a disruption of redox signaling and regulation and/or molecular damage"**[25].** Oxidative stress is the leading cause of liver and kidney damage **[26]**. The potential antioxidant properties of melatonin may be related to **a)** The Cascade antioxidant signaling independent receptor, such as Cyclic3-hydroxymelatonin (C3OHM), N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), and N1-acetyl-5-methoxykynuramine(AMK)**[27].**

b) Inflammatory processes, including activator protein 1 (AP-1); hypoxia-inducible factor 1α (HIF-1α); nuclear factor erythroid 2 factor 2 (Nrf2); nuclear factor kappa B (NF-κB)**[28]. c)** The apoptotic system; B-cell lymphoma 2 (Bcl-2)**[29] d)** oxidative system stability, including reactive oxygen species (ROS) **[28].** All these processes are linked to an increase in enzymatic substances such as SOD, GPx, and CAT and a decrease in the lipid peroxidation marker MDA **[30].** Melatonin is the body's key antioxidant, reducing oxidative stress and preventing liver and kidney damage, which in intern was lacking when the pineal gland was removed. **[31-33]**. The increase in pinealectomy oxidative stress of the liver and

kidney **[34]** including MDA, is attributed to a decrease in the liver and kidney antioxidants, including SOD, CAT, GPx, GSH, and an increase in GST due to low melatonin levels in the pinealectomy group**[35].** The liver toxicity was corroborated by the findings of liver functions, which revealed an increase in the activity of ALT, AST, and ALP, as well as a decrease in the concentration of albumin and total protein. **[35]**. However, the kidney toxicity is confirmed by the findings of the kidney functions, which reveal an increase in the content of creatinine, urea, and uric acid. **[34]**. These changes in liver and kidney also were verified by histopathological investigations. Pinealectomy in cholestatic rats worsens liver fibrosis, ductular response, and biliary injury. This is connected to lower amounts of melatonin, which generally reduces oxidative stress and inflammation. Melatonin deficiency increases reactive oxygen species (ROS) and upregulates profibrotic markers such as collagen type I alpha 1 (Col1a1) and fibronectin 1 **[36]** which is confirmed by the current study as pinealectomy caused portal vascular dilatation, biliary proliferation, peri-portal round cells aggregation, lymphatic and lympho-vascular dilatation in addition to, focal portal fibroplasia and many hepatocellular apoptosis**[37].** Melatonin deprivation due to pineal dysfunction also promotes renal tubular vacuolization, necrosis, and inflammatory infiltration. Melatonin generally reduces lipid peroxidation and neutrophil formation in renal tissues **[38].**

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