Research Article

Biochemical and Histological Effects of Long-Term Administration of Estrogen on Female Mice

Talal Jabal Hussen[1], Sattar J.J. Al-Shaeli[1]*, Baidaa H.R. Al-Mahna[2], Hasanain A.J. Gharban[3]

[1]Department of Basic Sciences, College of Dentistry, University of Wasit, Iraq; [2]Department of Veterinary Public Health, College of Veterinary Medicine, University of Wasit, Iraq; [3]Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Wasit, Iraq.

Abstract | Estrogen is a steroid sexual hormone responsible for developing of female characteristics, treatment the symptoms of menopause, and as a replacement therapy for postmenopausal with controversial topic. Investigation the long-term effect of estrogen administration on some biochemical markers and histological structure of the liver, spleen and kidney. An overall 40 BALB/c adult female mice were selected, acclimated, and divided randomly and equally into control (non-received estrogen) group and estrogen group. After 35 days of estrogen therapy, blood samples and tissue sections were collected from all study animals for biochemical assays and histology detection. The obtained data show that the biochemical markers displayed significant increased in estrogen administration mice compared to control. These markers are including alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (Cr), and malondialdehyde (MDA). Whereas, administration estrogen to mice caused significant dropdown the activity of antioxidant markers including catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) compared to control mice. Histologically, tissue sections of liver, spleen and kidney in mice exposed to estrogen showed variable degrees of abnormalities in their histological architecture and textures in compared to normal mice. This study is unique as it estimates the influencing of the long-term administration of estrogen on several biochemical markers and the histological alteration of liver, spleen, and kidney. Nevertheless the obtained result, further specific qualitative and quantitative research is needed to provide more details about the impact of estrogen on various body organs.

Keywords | Estradiol, Hepatotoxicity, Renal toxicity, Spleen, Antioxidant, Iraq

Received | April 20, 2024; **Accepted** | May 22, 2024; **Published** | June 27, 2024
***Correspondence** | Sattar J.J. Al-Shaeli, Department of Basic Sciences, College of Dentistry, University of Wasit, Iraq; **Email:** salshaeli@uowasit.edu.iq
Citation | Hussen TJ, Al-Shaeli SJJ, Al-Mahna BHR, Gharban HAJ (2024). Biochemical and histological effects of long-term administration of estrogen on female mice. Adv. Anim. Vet. Sci., 12(8):1563-1572.
DOI | https://dx.doi.org/10.17582/journal.aavs/2024/12.8.1563.1572
ISSN (Online) | 2307-8316

INTRODUCTION

Estrogens are specific steroid hormones which exert their biological effects across numerous organ systems (Patel *et al*., 2018). Although, there are three naturally distributed estrogens including estriol, estrone, and estradiol; the last is the most well-known active and predominant form in post-pubertal and non-pregnant females (Tang *et al*., 2022). In adult females, circulatory estradiol is primarily synthesized in the ovaries throughout the menstrual cycle to gradually rise at the first and second stages of follicular phase, and decreased until the luteal phase (Haryanti, 2023).

Much like the other steroid hormones, estradiol passively diffuses into the cell, binds to cytosolic estrogen receptors (ERs) and activates or suppresses the genomic and/or non-

genomic pathways (Mamnoon *et al*., 2020; Gonçalves *et al*., 2023). Although, the genomic pathway spans over a course of hours, estrogens can have more rapid course of action via the non-genomic pathway and acts within seconds or minutes (Balbi *et al*., 2019). This can occur through ERs which are situated within or underneath the cellular plasma membrane, or it can occur along related proteins of non-ER plasma membrane (Ma *et al*., 2021). However, activation of these pathways leads to various intracellular responses such as increases in intracellular calcium (Ca^{2+}), nitric oxide and activation of protein kinases (Cyrus *et al*., 2021; Sheppard *et al*., 2022).

The physiological effects of Estrogen can occur through activation of ERs, in particular ER-alpha (ER-α) and ER-beta (ER-β), which related to plasma membrane, cytoplasm, and nucleus in cells of vascular smooth muscles, cardiomyocytes, and vascular endothelial cells among the cardiovascular system of mammalians which all regulated physiologically through the normal action of estrogen and its receptors (Carbajal-García *et al*., 2020). Thus, abnormal estrogen function and impairment of its receptors can contribute to several pathologies including asthma, cardiac ischemia, rising blood pressure, and more cardiac pathology (Carbajal-García *et al*., 2020). Also, estrogen regulates physiological functions of numerous organ systems by expression of ER-α in uterus, mammary gland, ovaries, male reproductive system, prostate, liver and skeletal homeostasis; in addition to expression of ER-β in immune cells, ovary, prostate and the liver, therefore dysfunction of estrogen and its receptors can lead to develop several organs specific implication like female reproductive diseases including infertility, ovarian cancer, endometriosis, poly cystic ovary, and more pathologies (Tang *et al*., 2019; Roma and Spagnuolo, 2020). Also, both ER-α and ER-β are expressed throughout the brain, spinal cord, heart, blood vessels, adipose tissue and lung (Torres Irizarry *et al*., 2022).

On the other hand, several epidemiological studies and clinical observations have linked estradiol to the causes and incidence of chronic inflammation that caused several diseases and to impair bacterial clearance by reducing antimicrobial peptide secretion, impairing neutrophil function and blunting the immune response of pro-inflammatory reaction (Kanda *et al*., 2019; Nusbaum *et al*., 2020; Schwartz *et al*., 2023). Recently the liver dysfunction due to administration of estrogen or its components has bringing attention due to developed several disorders related to function of the liver which lead to gallstones cholesterol formation and incidence of hepatic malignancy (DeLeon *et al*., 2020; Ezhilarasan, 2020). Similarly, spleen shows several alterations in response to estradiol including increase proliferation of both monocyte and phagocyte cells associated with increased their activity. Furthermore, the macrophages in red pulp of splenic cord showed high cellular number and size (Weng *et al*., 2020; Pandey *et al*., 2022). The risk increasing of new kidney damage and negatively affecting females with abnormal kidney function were recorded as a result of estrogen treatment (Sabbatini and Kararigas, 2020). Although, the previous limited studies showed various impact of estradiol on specific organs, however, the precise effects of administration of estrogen still not elucidate completely and required further investigation. Therefore, this study aimed to investigate the long-term effect of estrogen administration on several liver, kidney, antioxident biochemical markers to determine whether the estrogen possesses negative role on ALT, ALP, AST, BUN, Cr, CAT, GSH-Px, SOD, and MDA markers. Furthermore, identify whether the estrogen exhibits specific histological alteration of the liver, spleen and kidney architectures.

MATERIALS AND METHODS

Animals

An overall 40 BALB/c adult female mice were obtained from local market. BABL/c are inbreed mice that extensively used in wide range of research aspects due to no variable and genetically established with strongly expressed of cellular estrogen receptors (Tam and Cheung, 2020). The age and weight of the mice were recorded as ≤4 months and 25-40 gm respectively. The study mice were acclimated to their environments for 1 week in the house of animal (Veterinary College, Wasit University). The mice during acclimation were exposed to standard condition including light, food and water availability.

Study design

After acclimation, the mice were randomly distributed into two groups; control that does not received estradiol, and experimental that received estradiol, housed individually in each cage and continued at the same acclimating conditions for eating, drinking and housing. The 17β-estradiol (Sigma-Aldrich, USA) was prepared according to manufacturer recommendation using ethanol and distilled water as stock solution and stored at -20 °C until use. The working solution of 17β-estradiol was administered to mice of experimental group for 35 days following the peroral method using 56 µg / kg body weight daily (Ingberg *et al*., 2012).

Samples

At the terminal of the experiment, all study mice were injected with 0.3 mg/kg ketamine and 0.1 mg/kg lidocaine, and blood samples were directly collected via heart puncture using a disposable syringe. The blood samples collected into free-anticoagulant glass-gel tubes (AFCO, Jordan), and centrifuged for 15 minutes at 5000 rpm. Then the sera were aspirated and inserted into new Eppendorf

labeled tube and stored at 4ºC prior to use for estimating serum biochemical markers.

Furthermore, after blood collection, all study mice were euthanasia by cervical dislocation. The mice were dissected, and tissue sections of the liver, spleen and kidney of each study mouse were collected in plastic containers filled with 10% neutral buffered formalin (NBF) for fixation. All tissue samples were sent to laboratory for processing within 24 hours of sectioning.

Biochemical assays

According to the manufacturer working guides, SunLong Biotech, China ELISA kits were used to quantify the alanine aminotransferase [ALT (Cat No: SL00229Mo)], alkaline phosphatase [ALP (Cat No: SL0031Mo], aspartate aminotransferase [AST (Cat No: SL0086Mo)], blood urea nitrogen [BUN (Cat No: SL0892Mo)], creatinine [Cr (Cat No: SL0159Mo)], catalase [CAT (Cat No: SL0747Mo)], glutathione peroxidase [GSH-Px (Cat No: SL0241Mo)], superoxide dismutase [SOD (Cat No: SL1341Mo)], and malondialdehyde [MDA (Cat No: SL0051Mo)]. The processed and values of optical density (OD) of each kit were measured by the Automated Microplate Photometer (BioTek, USA). Then, concentrations of each marker in study samples were calculated in the standard curve based on concentrations of Standard Solution of each kit.

Histology

Briefly, the formalin fixed tissue sections of liver, spleen and kidney were dehydrated by ethanol, cleared by xylene, infiltrated and embedded by paraffin, sectioned by microtome at 4-5 µm thickness and mounted on the slides to be stained later with the Hematoxylin and Eosin following the manufacturer instructions (Syrbio, Syria). The stained slides were visualized under an objective lens of 10× of light microscope (MEIJI, Japan), (Gharban *et al.*, 2023).

Data analysis

The Microsoft Office Excel (version 2019) was implicated to data documentation. The GraphPad Prism Software (version 8) was used to analyse the data statistically by applying the t-test analysis of variance (Al-Sarray and Al-Shaeli, 2023). The Differences between the values of study groups were considered significant at $P<0.05$. The data were displayed as Mean±Standard Error of Mean (M±SEM), (Al-Shaeli *et al.*, 2022).

RESULTS AND DISCUSSION

Biochemical markers

Interestingly, the mice that received estrogen for 35 days displayed various significant results of measured markers than the control. The levels of liver markers including ALT, ALP, and AST were significantly elevated in estrogen treated mice compared to control (Figures 1, 2, 3). The recorded percent of increases were 102% ± 4.75%, 47.6% ± 4.1%, and 198.7% ± 6.1%, respectively.

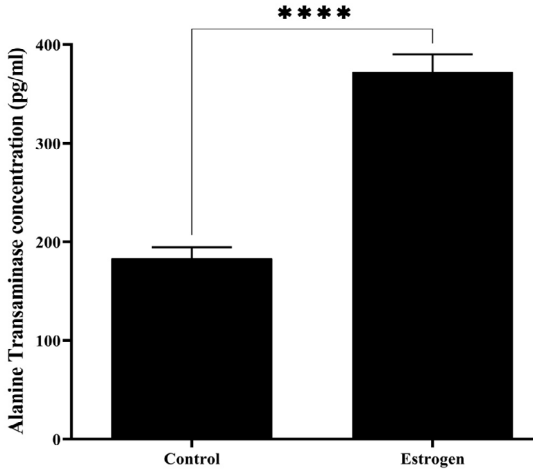

Figure 1: Estrogen administration increases the concentration of ALT enzyme.

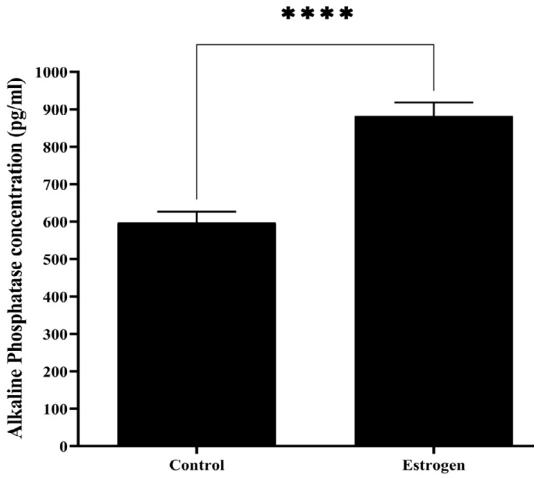

Figure 2: Estrogen administration increases the concentration of ALP enzyme.

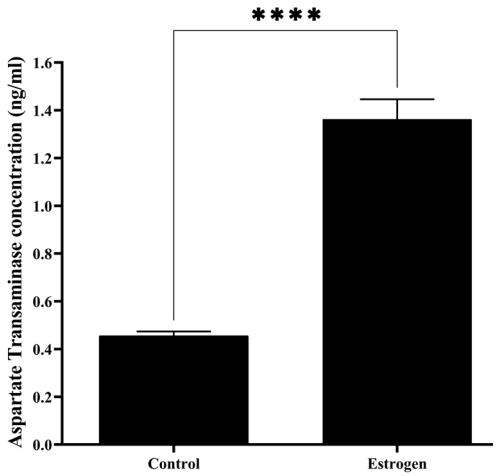

Figure 3: Estrogen administration increases the concentration AST enzyme.

Furthermore, the levels of kidney markers including BUN and Cr were also significantly elevated in estrogen treated mice compared to control (Figures 4, 5). The recorded increases were 244.4%±4.5% and 126.8%±4.4%, respectively.

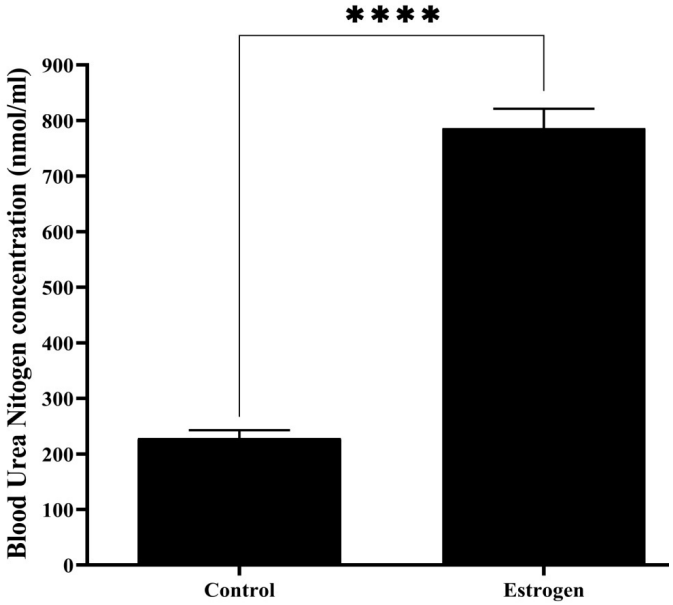

Figure 4: Estrogen administration increases the concentration BUN.

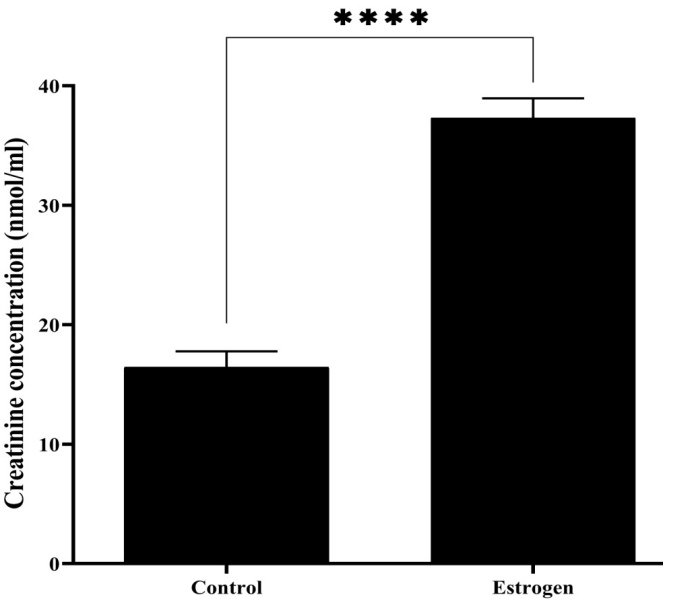

Figure 5: Estrogen administration increases the concentration Cr.

The measured antioxidant markers including CAT, GSH-Px, and SOD were significantly dropdown in estrogenic exposure mice compared to control (Figures 6, 7, 8). The recorded decreasing percent were 24%±4.1%, 26.3%±1.7%, and 59.9%±11.8% respectively. While, measured oxidant marker MDA showed significant elevation in mice exposed to estrogen than the control (Figure 9). The recorded increasing value was 82%±2.5% in mice received estrogen.

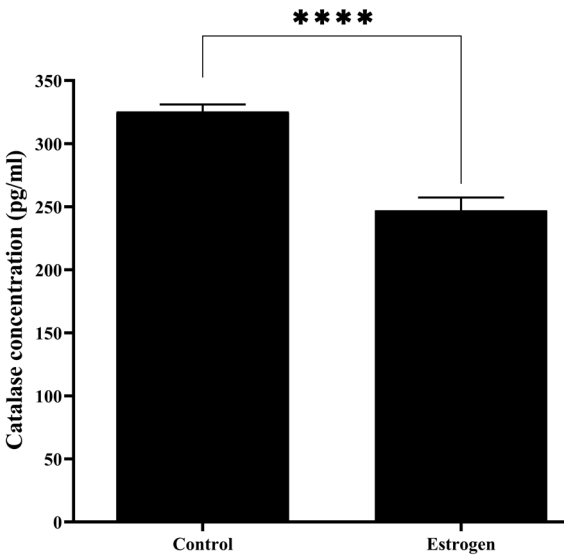

Figure 6: Estrogen administration decreases the concentration of CAT.

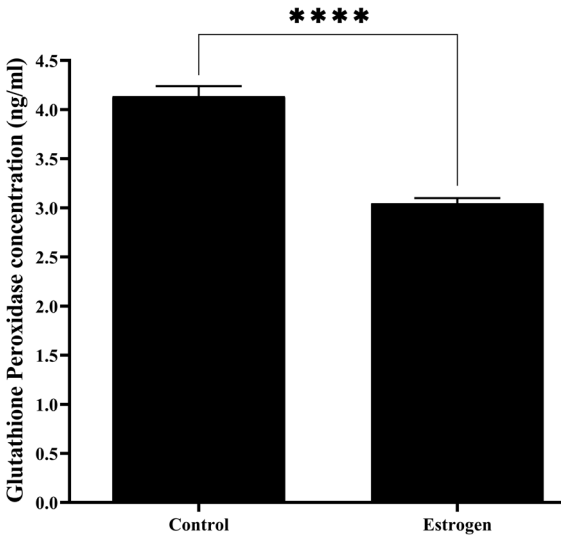

Figure 7: Estrogen administration decreases the concentration of GSH-Px.

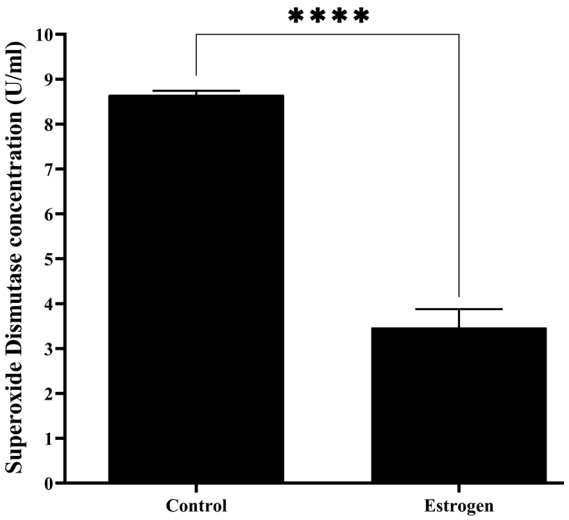

Figure 8: Estrogen administration decreases the concentration of SOD.

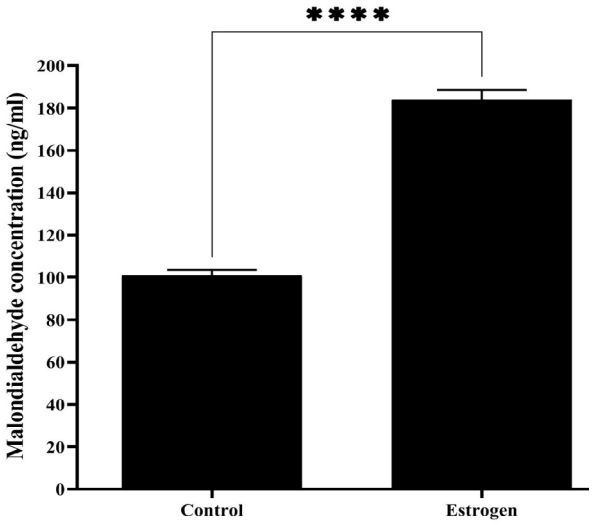

Figure 9: Estrogen administration increases the concentration MDA.

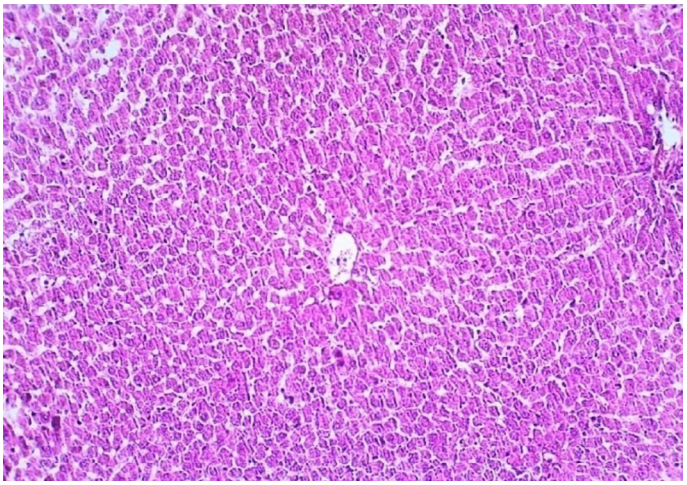

Figure 10: Normal histological structure of liver in control group mice. The prepared section was stained with Hematoxylin and Eosin stain and examined at 10X. The image is represented experimental mice.

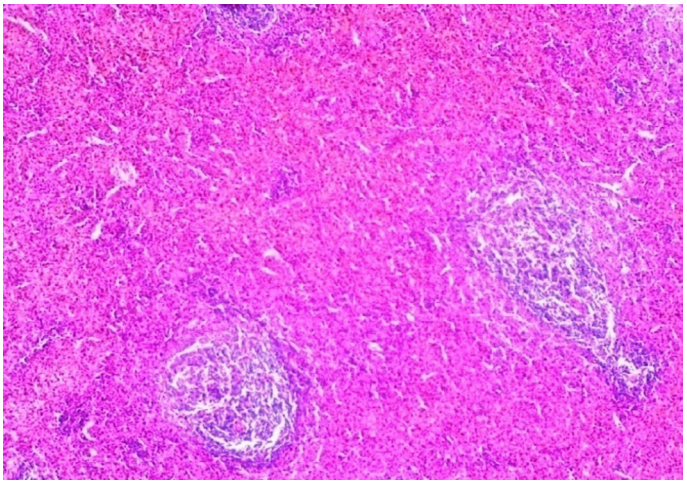

Figure 11: Normal histological structure of spleen in control group mice. The prepared section was stained with Hematoxylin and Eosin stain and examined at 10X. The image is represented experimental mice.

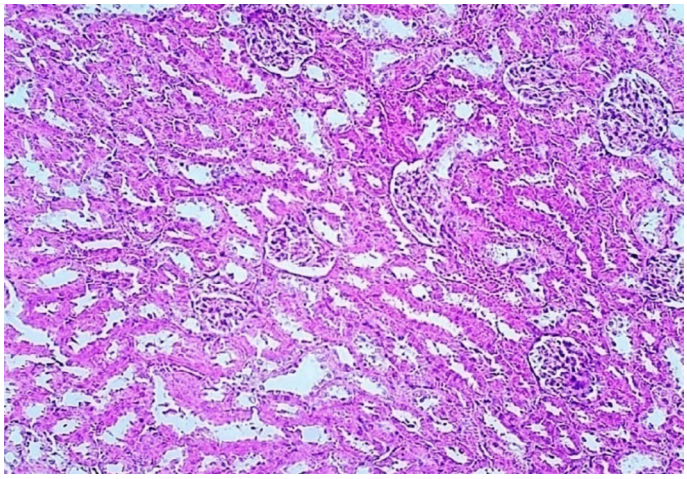

Figure 12: Normal histological structure of kidney in control group mice. The prepared section was stained with Hematoxylin and Eosin stain and examined at 10X. The image is represented experimental mice.

Histology

In control mice groups, the histological architecture of examined organs including liver, spleen and kidney were appeared normal with absence of any abnormalities. The liver displayed normal size, structure, and arrangement of hepatocytes as a cord around central vein (Figure 10). Similarly, the histological structure of the spleen in control mice showed normal structure and distribution of lymphocytes that form red and white pulp (Figure 11). Furthermore, the histological structure of kidney in control mice displayed normal glomerulus, masengial cells, renal tubules, and cellular structure of these components (Figure 12). However, several pathological changes were identifying in the tissue sections of selected organs in mice that received estrogen for 35 days. The liver of estrogenic mice showed severely fatty degeneration, aggregation of mononuclear inflammatory cells, droplet infiltration in cytoplasm of hepatocytes, dilation of sinusoidal spaces, deposition of fibrin in blood vessels, damaging of parenchyma, thrombus formation and congestion of hepatic veins, amyloidosis, and cloudy swelling of parenchyma (Figure 13). Furthermore, the spleen of mice exposed to estrogen exhibited different degrees of amyloidosis, blood vessels congestion and hyperplastic changes (Figure 14). Moreover, in kidney sections of estrogenic mice, there was an obvious damaging in proximal tubules and veins, fibrosis in parenchyma, narrowing in ureter, degenerative changes and narrowing in renal tubules, moderate glomerular tuft atrophy, congestion in renal veins, and increasing in glomerular (Figure 15).

Due to wide spectrum of clinical application of estrogen as a contraceptive and relief the symptoms of post menopause, it recently considered as a public debate, thus brings attention for extensive research (Naftolin *et al.*, 2019; Palacios *et al.*, 2019). Rodent in particular mice are

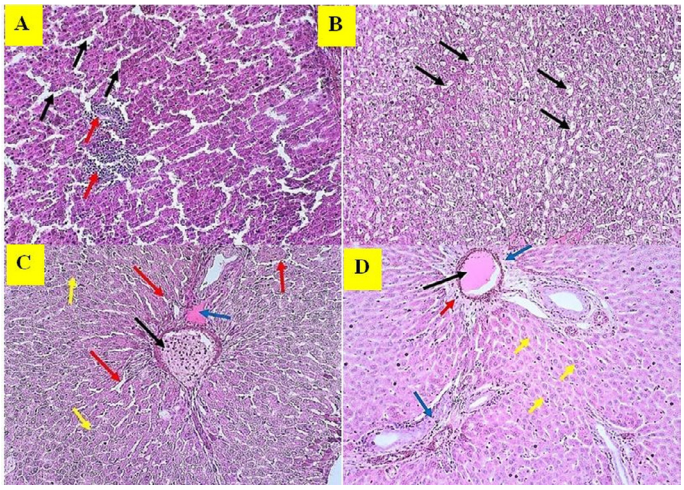

Figure 13: Histopathological changes of liver in mice received estrogen. (A): Sinusoidal dilation (Black arrow) and infiltration of mononuclear cells (Red arrow); (B): Droplets of cytoplasm (Black arrow); (C): Congestion of veins (Black arrow), Amyloidosis (Blue arrow), Sinusoidal space (Red arrow), Degeneration of parenchyma (Yellow arrow); (D): Thrombus (Black arrow), Deposition of fibrin (Blue arrow), Perivascular cuff (Red arrow), Degeneration of parenchyma (Yellow arrow). The sections were stained with Hematoxylin and Eosin stain, examined at 10X, and the images are represented experimental mice.

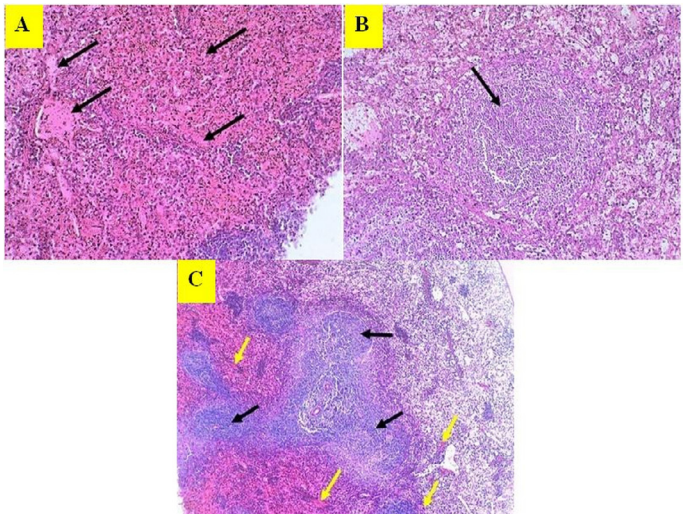

Figure 14: Histopathological changes of spleen in mice received estrogen. (A): Amyloidosis (Black arrow); (B): Hyperplasia (Black arrow); (C): Hyperplasia (Black arrow), Congestion of blood vessels (Yellow arrow). The sections were stained with Hematoxylin and Eosin stain, examined at 10X, and the images are represented experimental mice.

specific models that used to elucidate the precise biological mechanism of the role and effect of estrogen (Mittelman-Smith *et al*., 2017; Rauner *et al*., 2021). However, the level of estrogen in the body and administration doses are the main factors that involved in exerting its various effect, therefore it is essential to validate the doses use to identify the effect of estrogen (Goodman, 2012; Ruan and Mueck, 2022). It is clearly that the human is received the estrogen per os, thus this route of administration of this hormone is particularly desirable in animal model due to several reasons including pharmacokinetics (Gordon *et al*., 1986; Ingberg *et al*., 2012; Battipaglia *et al*., 2023). In this study, the peroral administration of exogenous estrogen (17β-estradiol) was identified a significant impact on biochemical markers and histology changes of selected examined body organs. However, the effect of this hormone on the body function still debated and depends on gender (Farahmand *et al*., 2021).

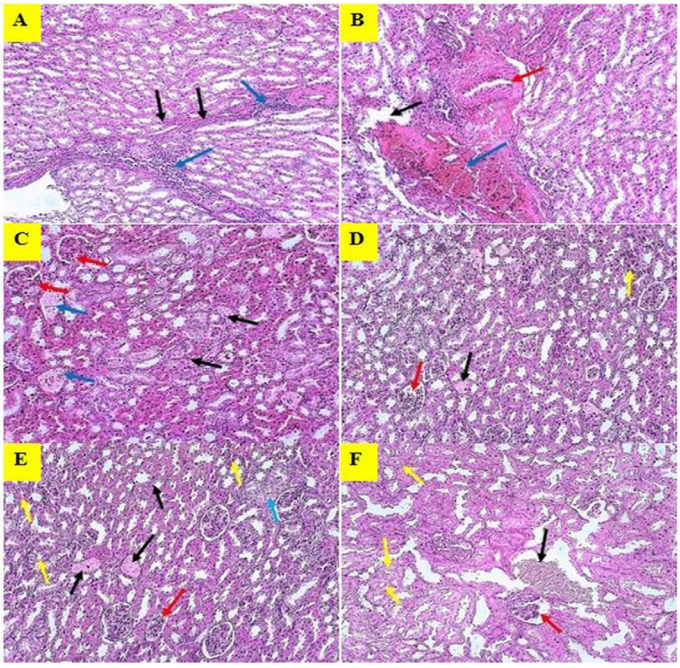

Figure 15: Histopathological changes of kidney in mice received estrogen. (A): Damaging of proximal tubules (Black arrow) and Inflammatory cells infiltration (Blue arrow); (B): Damaging of venous wall (Black arrow), Venous congestion (Blue arrow), Narrowing of ureter's lumen (Red arrow); (C): Amyloid degeneration (Black arrow), Amyloid infiltration in veins (Blue arrow), Amyloid infiltration in proximal tubules (Red arrow); (D): Congestion of veins (Black arrow), Atrophy of glomerular tufts (Red arrow), Hypertrophy of proximal tubules (Yellow arrow); (E): Congestion of veins (Black arrow), Parenchymal fibrosis (Blue arrow), Atrophy of glomerular tufts (Red arrow), Hypertrophy of tubular lumen (Yellow arrow); (F): Congestion of veins (Black arrow), Hypertrophy of glomerular space (Red arrow), Hypertrophy of tubular lumen (Yellow arrow). The sections were stained with Hematoxylin and Eosin stain, examined at 10X, and the images are represented experimental mice.

Worldwide, several studies have been denoted the role of estrogen in improving and protecting the function of body organs (Kummer *et al*., 2011; Petrica *et al*., 2012; Park and Kim, 2018); nonetheless, little known about the adverse effects. Elevation of liver markers including ALT, ALP, and

AST in responses to 17β-estradiol was seen in the current study. In comparison with that detected by different authors who administered estradiol in different clinical cases, the findings were showed a significant variation in their values. Yin *et al.* (2000) observed that estrogen is participated in rat liver damage due to increasing the levels of ALT. Xu *et al.* (2002) showed that the ALT and AST enzymes concentrations were not changed after using of estrogen in CCL4-induced liver fibrosis in rats. Pinheiro *et al.* (2007) revealed the increasing levels of serum AST and GGT after estradiol administration in patients with turner syndrome. Ahmed and Hassanein (2012) found that the administration of estradiol to male rats for 15 days causes a significant enhancement in concentrations of GSH-Px and decreases in MDA, ALT and AST. Kowalska *et al.* (2018) concluded the higher ALT, AST and GGT activities with changes in lipid profile after oral contraceptives. The possible reason behind adverse effect of estrogen could be high level of circulating estrogen may disrupt the estrogen receptors and therefore induces liver dysfunction and increases ALT, ALP, and AST. Thus, the study suggested that the long term elevated level of estrogen in the body could induces several illness particularly liver diseases and therefore, maintaining physiological level of estrogen is crucial to prevent prevalence of liver dysfunction.

Several recent studies data displayed that the estrogen possesses an important role in regulation of kidney physiological function (El-Gendy *et al.*, 2019; Darvishzadeh *et al.*, 2021; Thomas and Harvey, 2023). Nonetheless, Ma *et al.* (2021) mentioned that the administration of estrogen to wild type mice could cause kidney injury and developed lupus phenotype which resulting in increased expression of high proportion of ERα in immune cells. In this study, significant elevation in values of BUN and Cr indicate that either kidneys aren't working well or there is a higher production of urea in the liver in case of urea; and reducing of blood flow or losing of body fluid in case of creatinine. It is clearly that the physiological level of estrogen possesses renal protective role. However, high level of circulating estrogen could impaired cellular renal function through impairment of estrogen receptors and induces several kidney damages, which is the possible cause of increasing BUN and Cr level. Accordingly, the study suggested that the renal toxicity in response to long term exposure to estrogen could be prevented by maintaining the proper physiological level of estrogen, while estrogen administration to individuals with several renal impairments could attenuate the impairment and improved kidney functions.

In animal normal cases, many studies provided the evidence that estrogen possesses incredible role to rise the antioxidant activity and genes related to longevity (Abdelrazek *et al.*, 2019; Delgobo *et al.*, 2019) and human (Kong *et al.*, 2019; Borrás *et al.*, 2021). These effects may be attributed to the short-term using of estrogen, type of estradiol, as well as dose and route of administration. In addition, almost studies have carried out in ovariectomized animal models or in women suffering reproductive problems; so that, estrogen levels being at normal or subnormal levels. The using of estrogen in normally females could produce diverse negative effects as a result of abnormal higher blood estrogens to reflect their impacts on various boy systems. Accordingly, the current result showed reduction in antioxidant activity and elevation of oxidant activity in response to estrogen. This diverse effect of estrogen could be due to the increases circulatory level of estrogen that induced cytotoxicity and lead to increase the production of free radicals associated with inhibition of antioxidant enzyme activity. Based on that, the study suggested that the long term increases in the level of estrogen could impaired the oxidant/antioxidant activity resulting in elevated of free radicals that involving in initiation of various pathogenesis.

Yin *et al.* (2000) found that the increasing weight of liver, steatosis, severe panlobular fatty accumulation, were not treated with using of estrogen that causes additional disorders such as leukocyte infiltration and hepatocytic necrosis. Aiyer and Gupta (2010) recorded that the administration of estradiol causes in increasing the weight of liver. Ahmed and Hassanein (2012) found that the administration of estradiol to male rats for 15 days resulted in treatment of injured islets of pancreas that caused by STZ-induced diabetes, proliferation of Kupffer cells, hepatocytic vaculation, and lymphocytic infiltration.

In an experimental study in rats, Abd El-Lateef *et al.* (2019) reported that kidney damage characterized by increasing interstitial edema, intra-tubular damage, infiltration of inflammatory cells, necrosis of proximal tubules, widened bowman's spaces and scattered atrophied glomeruli were alleviated obviously due to using of estradiol benzoate for 1 week. Darvishzadeh *et al.* (2021) identified that the administration of estrogen could induced damage of the kidney and this adverse effect of this sex hormone is various according gender variance. El-Gendy *et al.* (2019) detected the main histopathological changes in the kidney of rat when exposed to estrogen that could cause renal endocrine dysfunctions included congestion glomerular capillaries, cytoplasmic vacuolation, losing of apical brush border, large clear lumen, and appearance of eosinophilic cast in some of the lumen.

In spleen, amyloidosis is the most abundant symptom observed in tissue sections observed by light microscope. Splenic amyloidosis is usually asymptomatic but it may be accompanied by hepatic disorders such as abnormal liver function (Dias *et al.*, 2023). Therefore, the incidence of

this lesion might be caused by direct effect of estrogen on spleen or indirectly by hepatic disorders.

CONCLUSIONS AND RECOMMENDATIONS

This study is presented unique data involving in estimates the influencing the long-term administration of estrogen. Given the role of estrogen in preventing, causing and exacerbating disease a good knowledge of how estrogens are synthesized and metabolized may help in the understanding and treatment. Fundamentally, the physiological level of estrogen participated in maintaining several normal body function activity including cellular growth and homeostasis, and metabolism. Furthermore, it exhibited regulatory and cellular protective roles against pathogenesis in various body organs. However, estrogen toxicity is resulting from long term of circulating estrogen molecule in the body which could participate in development various pathologies in susceptible organs of the body. With respect to proper role of physiological level of estrogen, significant abnormalities in measured markers and tested tissue sections of liver, spleen and kidney necessitated furthermore studies on the impact of estrogen on different body organs. In addition, knowledge about how estrogen produces these effects is important and required for support. Therefore, specific qualitative and quantitative studies are in needs to determine the proper and precise effect of estrogen and main signaling pathway of the effect under molecular determination.

Study limitations

The research aim and objectives acknowledged uncontrolled hormonal changes of female mice during the study which required specific and precise further investigation. Furthermore, the research acknowledged limited self-fund and availability of specific molecular techniques to provide insight signaling of estrogen on various organs in the body. These limitations can overcome through availability of funding resource and also availability of instruments and techniques including molecular gene expression PCR, Western blot, and immunohistochemistry.

ACKNOWLEDGMENTS

The authors are acknowledging the assistant of animal house staff in the Veterinary Medicine College (University of Wasit) during this work.

NOVELTY STATEMENT

Maintaining the physiological level of circulating estrogen is fundamental in cellular regulation and normal body function. Several studies investigated the role of estrogen in various organs function as hormonal replacement or treatment on different and specific pathology. However, the impact of excessive circulatory estrogen for prolong period on body organs function is not elucidate completely. Therefore, the current study provided unique data that showed the excessive estrogen could dysregulated the estrogen receptors and impaired the cellular function of liver, kidney, and spleen through alteration of oxidant/ antioxidant activity and induced cytotoxicity in normal organs in female body.

AUTHOR'S CONTRIBUTION

Talal Jabal Hussen: Collection and processing of tissue sections. Sattar J.J Al-Shaeli: Examination of tissue sections. Baidaa H.R. Al-Mahna: Biochemical assays of liver enzymes. Hasanain A.J. Gharban: Biochemical assays of kidney enzymes and antioxidants, and statistical analysis of obtained data. The authors read this manuscript and approved it as a final version to submit for publication.

Funding

No external funds were received (private funds only).

Ethics approve

The current study is licensed by the Scientific Committee of both Dentistry and Veterinary Medicine collages at University of Wasit.

Author's declaration

The authors declare that the manuscript is submitted to publish in Slovenian Veterinary Research Journal and declare that neither the whole manuscript nor its part of content have been published or submitted for publication in any journal or magazine for privet or public distribution.

Ethics approve and participant consent

This study was performed under a license of Scientific Committees of Dentistry and Veterinary medicine Colleges, University of Wasit.

Study animal details

Total 40 BALB/c adult female mice with ≤4 months and 25-40 gm age and weight were used in the study as 20:20 control and estradiol treatment for 35 days. Mice were maintaining in cages with free access to food and water in the house of animal (Veterinary College, Wasit University) during the study and acclimation time at the standard condition. After experimental time, the samples were collecting from mice after exposed to chloroform to euthanize the animals.

Availability of data and materials

All obtained and analyzed data in this study are including

in this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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