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Regulation of the adverse effects of 2,4-dichlorophenoxyacetic acid and gamma-irradiation in rats by some dietary oils

Mamdouh M. T. Eassawy[1] and Amel F. M. Ismail[2*]
[1] Regional Center for Food and Feed (RCFF), Agricultural Research Center (ARC), Giza, Egypt.
[2] Drug Radiation Research Department, Biotechnology Division, National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, Cairo, Egypt.
[*]Corresponding Author: afmismail@gmail.com

ABSTRACT

2,4-dichlorophenoxyacetic acid (2,4-D) is a synthetic herbicide. It provokes lipid peroxidation (LPO), oxidative stress, inflammation, and apoptosis in mammalian cells. The purpose of this study is to investigate the regulatory effects of flaxseed oil (FSO) and fish oil (FO) against the combined toxicity of 2,4-D and gamma-radiation (2,4-D/R) in rats' liver, kidney, testis, and brain tissues. Eight groups of ten rats each were organized as follows: Group 1: Control (C), Group 2: FSO, Group 3: FO, Group 4 (R): rats were exposed to gamma-radiation (2 Gy per week for 4 weeks), Group 5(2,4-D): rats were administered 2,4-D, Group 6 (2,4-D/R): rats were administered 2,4-D, followed by exposure to 2 Gy of gamma-radiation per week for 4 weeks, Group 7: FSO/2,4-D/R treated rats, Group 8: FO/2,4-D/R treated rats. 2,4-D/R treated rats demonstrated significant alterations in the activity of the hepatic enzymes (ALT, AST, ALP, and LDH), the levels of urea, creatinine (Creat), total cholesterol (TC), triglycerides (TG), total protein (TP), albumin (ALB), reproductive hormones (Testosterone (TH), Follicle Stimulating Hormone (FSH), and Luteinizing Hormone (LH)), cytokines (IL-1β, IL-6, TNF-α, NF-κB), acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) enzymes in serum. In addition, 2,4-D/R intoxicated rats showed significant changes in the levels of malondialdehyde (MDA), glutathione reduced (GSH), and trace elements (Ca^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+}), as well as significant alterations in the gene expression ratios of the apoptotic markers (BAX, BCL2, cytochrome c, caspases-9, and caspase-3) in the liver, kidney, testis, and brain tissues. Moreover, 2,4-D/R demonstrated lower sperm count and motility. FSO and FO-treated 2,4-D/R groups exhibited significant amelioration in the changes in the investigated parameters. The histopathological investigation confirmed the biochemical investigations. In conclusion, FSO and FO showed regulatory effects on 2,4-D/R toxicity-induced damage to liver, kidney, testis, and brain, via regulation of mitochondrial apoptotic pathway and trace elements' levels.

Keywords: Flaxseed oil; Fish oil; oxidative stress; inflammation; mitochondrial apoptosis.

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INTRODUCTION

Agricultural productivity has to meet the increasing global food supply demands caused by population growth, climate changes, and economic issues. So, agricultural activities must be enhanced and expanded **(Hemathilake and Gunathilake, 2022, Muhammad et al., 2023)**. Herbicides are used worldwide to suppress weed growth and boost agricultural yields. However, the widespread and ongoing use of pesticides and herbicides can eliminate various valuable floras, causes pollution and detrimental effects on the ecosystem **(Islam et al., 2018)**. Pesticides, herbicides, and their metabolites that comprise long half-life times and building up in the food

chain may have serious negative effects on animal and human health **(Jote, 2019, Singh et al., 2023)**. Due to its low cost, ease of use, efficiency in controlling weeds, and imitating auxin to promote plant growth, 2,4-dichlorophenoxyacetic acid (2,4-D) is a highly significant synthetic herbicide. These significant properties made 2,4-D widely exploited in many locations. Because of its high-water solubility, poor volatility, and minimal biodegradation, 2,4-D is often detected in water bodies around the world **(Jote, 2019, Muhammad et al., 2023)**. Nevertheless, it has been discovered that 2,4-D exposure and ingestion harms mammals, plants, and microbial populations **(Shafeeq and Mahboob, 2020, Shafeeq and Mahboob 2021)**. The hazardous properties of 2,4-D are caused due to its lipophilic nature and the dioxins moiety. In mammalian cells, this pesticide causes lipid peroxidation (LPO), oxidative stress, necrosis, and apoptosis **(Marouani et al. 2017)**. It also showed teratogenic and carcinogenic properties **(Jote, 2019, Marouani et al. 2017)**. In addition, even exposure to low doses, this herbicide causes some toxic effects in mammalian cells, such as endocrine disruption **(Garry et al., 2001)**, neuro-toxicity **(Gabraut and Philbert, 2002, Ferri et al., 2008)**, hepatotoxicity **(Tayeb et al., 2010, Shafeeq and Mahboob, 2021)**, nephrotoxicity **(Tayeb et al., 2012, Shafeeq and Mahboob, 2021)**, reproductive toxicity **(Gabraut and Philbert, 2002, Marouani et al., 2017, Zhang et al., 2017)**, and immune-toxicity **(Gabraut and Philbert, 2002)**. Some antioxidant compounds are investigated for their potential to reduce oxidative stress caused by pesticides. It has been shown that taurine, α-lipoic acid, β-carotene, vitamin C, vitamin E, and glucosinolates may all efficiently scavenge the free radicals produced by pesticides in animals **(Zeng et al., 2021)**. Natural oils extracted from natural plants, and marine organisms were used to recover the toxic effects of organophosphate insecticides **(Sadeghi et al., 2023)**.

Ionizing radiation (IR) is commonly used for medical, agricultural, and industrial purposes. However, there are a lot of damaging effects that restrict its applications **(Yue et al., 2023)**. The main factor responsible for these damages is the oxidative stress that occurs due to exposure to gamma rays. Oxidative stress is a state in which antioxidant defenses are undermined by unstable free radicals or reactive oxygen species (ROS), which may result in destruction of cellular constituents such as proteins, membrane lipids, and DNA **(Jit et al., 2022)**. One of the oxidative damage markers is the malondialdehyde (MDA) formation as an end-product of LPO **(Cheng et al., 2018)**. Exposure to gamma-irradiation provokes toxic effects in different organs, including hepatotoxicity **(Eassawy et al., 2021, Ismail et al., 2016b, Zaher et al., 2016)**, nephrotoxicity **(Ismail et al., 2016d & 2023, Salem and Ismail, 2021, Eassawy and Ismail, 2024)**, neurotoxicity **(Ismail and El-Sonbaty, 2016, Ismail et al., 2016a)**, and testicular toxicity **(Gawish et al., 2019)**. Cells have evolved their own antioxidant defense mechanism, which consists of both enzymatic and non-enzymatic components, to limit the potentially damaging effects of ROS. Moreover, the exposure to gamma-irradiation stimulates the inflammatory response in different tissues, mediated by activation of pro-inflammatory markers, including the cytokines like interleukin-1 beta (IL-1□), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), as well as activation of nuclear factor-kappa B (NF-κB) **(Eassawy et al., 2021, Ismail and El-Sonbaty, 2016, Ismail et al., 2016a, Ismail et al., 2016b, Ismail et al., 2023, Zaher et al., 2016)**. In addition, gamma-irradiation activates the programmed cell death or apoptosis in various tissues, via activation of caspases, leading to DNA fragmentation **(Salem and Ismail, 2021, Ismail et al., 2016b & 2016d)**. On the other hand, the antioxidant system inside the cells consists of low molecular-weight antioxidant molecules, such as glutathione reduced (GSH), melatonin, vitamins C and E, and various antioxidant enzymes **(Mirończuk-Chodakowska et al., 2018)**. In addition, the natural products that are rich in phytochemicals with high antioxidant activity show potential radio-protective activity on radiation damage during cancer radiotherapy,

through free-radical scavenging, suppression of oxidative stress, inflammation, and apoptosis as well as promoting the repair mechanism of the damaged DNA **(Zhang et al., 2023)**.

Mitochondria are important organelles that maintain the standard functional process, it is the main source of ROS, and also it is responsible for the eukaryotic oxidative metabolism **(Cui et al., 2023)**. Oxidative stress, inflammation, and apoptosis are normal defense mechanisms to stabilize the cells. They are correlated to each other. In the intrinsic apoptotic pathway, oxidative stress triggers apoptosis in different tissues. Apoptosis is a controlled programmed cell death that performs an essential task during the development, and aging, as well as during the progress of tumors and other diseases. It induces morphological modification of the cells due to activation of some apoptotic markers, leading to DNA damage. Oxidative stress and the released ROS down-regulate B-cell lymphoma 2 (BCl2) expression, and instigate mitochondrial membrane depolarization, leading to the up-regulation of the pro-apoptotic molecule kmBcl-2-associated X protein (BAX) gene expression, with an increase in BAX/BCL2 ratio. Activated BAX is translocated from the cytoplasm to mitochondria, inducing cytochrome c (Cyt C) liberation, which is localized to the mitochondrial inter-membrane area, improving the permeability of the outer mitochondrial membrane, thus enabling the transfer of Cyt C from the mitochondria to the cytoplasm. The released Cyt C is connected with the Apoptotic protease activating factor 1 (APAF-1) proteins, forming an apoptosome complex that stimulates caspases-9 activation. The activated caspases-9 induced caspases-3 in the mitochondria, through the intrinsic pathway of apoptosis. Then this activated caspases-3 initiates DNA fragmentation and leads to cell death **(Ott et al., 2007, Zhang et al., 2017)**. Oxidative stress can encourage an inflammation response through the activation of NF-κB. NF-κB demonstrates regulatory effects on some anti-apoptotic genes, leading to the activation of caspases cascades and directing the cells towards apoptosis **(Banik et al., 2021)**.

Flaxseed oil (FSO) is extracted from *Linium usitatissimum* plant seeds **(Bloedon et al., 2004)**. Alpha-linolenic acid (ALA), a valuable polyunsaturated omega- 3 fatty acid, is found and approximately performing 53% of FSO. ALA is the precursor of the other omega- 3 fatty acids family, docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3). Moreover, ALA controls some ailments **(Abdel Moneim et al., 2011, Kim et al., 2014)**. DHA is involved in building up the brain and promoting its function **(Ferreira Costa Leite et al., 2011)**. In addition, FSO contains seventeen percent linoleic acid (LA), nineteen percent oleic acid, three percent stearic acid, five percent palmitic acid, and one to four percent lignin **(Bernacchia et al., 2014)**. Consumption of FSO has beneficial health effects including anticancer, antiviral, antibacterial, anti-inflammatory, cardio-protection, anti-diabetic, immune-modulatory, ion reduction, and reduction of atherogenic risks. These effects are mainly recognized due to its ALA and the phenolic lignans **(Ismail et al., 2016c)**. Lignan is a phenolic compound. The human colon's bacterial flora transforms the lignan precursor secoisolariciresinol diglycoside (SDG) into enterodiol and enterolactone, the two main lignans found in mammals. Due to their strong capacity to scavenge free radicals, all of these lignans possess antioxidant properties **(Touré and Xueming, 2010)**.

Fish oil (FO) has high concentrations of omega-3 (ω-3), a polyunsaturated fatty acid (PUFA). It encompasses DHA, C22:6n-3, ALA, and EPA, C20:5n-3 **(Burdge and Calder, 2006)**. Single-cell marine organisms that are consumed by fish are responsible for the production of ω-3 FAs. These omega-3 fatty acids (ω-3 FAs) are potent antioxidants immuno-nutrients. They have anticancer **(Fabian et al., 2015, Freitas et al., 2019)**, and anti-inflammatory **(Wall et al., 2010)** effects. They also triggered apoptosis, and prevented proliferation in numerous malignancies **(Sun et al., 2009)**. In addition, omega-3 FAs can act synergistically with certain chemotherapeutic agents by enhancing the tumor radiosensitivity **(Wendel and Heller, 2009)**.

Omega-3 FAs have been extensively utilized in clinical pre-operative nutrition and have been shown to have hepatorenal protective effects **(Moussa et al., 2020, Saleh et al., 2020)**.

The purpose of this study is to investigate the regulatory effects of flaxseed oil (FSO) and fish oil (FO) against the combined toxicity of 2,4-D and gamma-radiation (2,4-D/R) in rats' liver, kidney, testis, and brain tissues.

MATERIALS AND METHODS

Chemicals

2,4-dichlorophenoxyacetic acid was obtained from LOBA Chemie Pvt. Ltd, India. FSO (cold pressed) obtained from Imtenan health shop, Cairo, Egypt. FO is a Seven Seas pharmaceutical package (Merk, UK).

Irradiation facilities

The Whole-body gamma-irradiation was performed at National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, Cairo, Egypt, using the Canadian Gamma Cell-40 (^{137}Cesium), manufactured by the Atomic Energy of Canada Limited, Ontario, Canada. Rats were exposed to 2 Gy/week for four weeks with an 8 Gy total radiation dose, and the radiation dose rate was 623 rad/s (radiation-absorbed dose per second) at the time of exposure.

Animals

Nile Pharmaceutical Co. (Cairo, Egypt) provided us with *Wistar* male albino rats (100-120 g). The animals were kept in specified laboratory environment suitable for animals' experiments, with a continuous availability of regular laboratory pellet diet and fresh tap water. The international guidelines for animal experiments were performed during this work, and *affirmed* by the NCRRT-Ethical Committee (60A/21).

Rats were treated orally by gastric intubation with 4 ml FSO/kg b.wt./day **(Shah et al., 2014)**, or with 4 ml FO/kg b.wt./day **(Denny Joseph and Muralidhara, 2012)**. However, rats were administered 2,4-D at doses of 150 mg/kg b.wt./day **(Tayeb et al., 2010)**.

Experimental design

Eight groups of ten rats each were organized as follows: Group 1: Control (C), Group 2: FSO, Group 3: FO, Group 4 (R): rats were exposed to gamma-radiation (2 Gy per week for 4 weeks), Group 5 (2,4-D): rats were administered 2,4-D, Group 6 (2,4-D/R): rats were administered 2,4-D, followed by exposure to 2 Gy of gamma-radiation per week for 4 weeks, Group 7: FSO/2,4-D/R treated rats, Group 8: FO/2,4-D/R treated rats.

Samples collections

After an overnight fasting period, following four weeks experimental period, all animals were anesthetized with 1.2 g urethane (Sigma–Aldrich, St Louis)/kg b.wt. **(Moheban et al., 2016)**. Serum was separated from the gathered blood samples.

The cauda epididymis was isolated and minced in physiological saline solution (0.9% NaCl, at 37 °C) to prepare the sperm suspension **(Faqi et al., 1998)**. Then, the free sperms (50 microliters) were examined and counted as millions/ml and their motility percentages were assessed as previously reported **(Majumder and Biswas, 1979)**, under a light microscope; the sperm motility percentage was calculated by applying: the sum of motile sperms that demonstrated forward movement × 100 related to the total sperms number.

Also, the liver, kidney, testes, and brain organs were dissected from each rat, submerged in ice-cold physiological saline, then washed with ice-cold de-ionized water, dried, and preserved at -80 ^{o}C.

The liver, kidney, testes, and brain tissues' sections were collected for the histopathological investigations. Ten percent neutrally buffered formalin was used to fix the tissue samples.

Following trimming, washing, and dehydration in increasing alcohol grades, the fixed specimens were cleaned in xylene, embedded in paraffin, sectioned at 4-6 µL thickness, and stained with hematoxylin and eosin (H&E) **(Bancroft and Layton, 2019)**.

Assessment of biochemical parameters

The biochemical parameters (ALT (Alanine Amino-Transaminase), AST (Aspartate Amino-Transferase), ALP (Alkaline Phosphatase), urea, creatinine (Creat), total cholesterol (TC), triglycerides (TG), total protein (TP), and albumin (ALB)) were determined in serum on biochemical blood analyzer (Alfa Wassermann Diagnostic Technologies, LLC, ACE, Alera, USA).

However, the serum lactate dehydrogenase (LDH) activity was assessed using the rat LDH ELISA kit (Elabscience Biotechnology Inc., Cat No. E-EL-R2547).

Hormonal investigations

Rat Elisa Kits were used for assessment of Testosterone (TH, Catalog No: CSB-E05100r CUSABIO, ng/ml), Follicle Stimulating Hormone (FSH, Catalog No: E-EL-R0391, Elabscience, ng/ml), and Luteinizing Hormone (LH, Catalog No: E-EL-R0026, Elabscience, mIU/ml).

Estimation of acetylcholinesterase and butyrylcholinesterase levels in serum

Acetylcholinesterase (AChE, Catalog Number: MBS725468) and butyrylcholinesterase (BChE, Catalog Number: MBS2700571) in serum were determined using ELISA kits for rats (MyBioSource).

Estimation of dopamine level in brain tissues

The dopamine (DA) levels were assessed in the brain tissues according to **Ciarlone (1978)**.

Estimation of malondialdehyde levels and glutathione reduced contents in different tissues

The liver, kidney, testis, and brain tissues' homogenates were prepared as described in **Eassawy et al., 2021, Salem and Ismail, 2021, Kaushik et al., 2018,** and **Elsonbaty and Ismail, 2020**, respectively.

The MDA levels, and GSH contents were assessed in different tissues using Biodiagnostic kits.

Determining the inflammatory markers by ELISA Technique in serum

Rat ELISA kits were utilized to measure the levels of the inflammatory markers.

Real-time quantitative reverse transcription– polymerase chain reaction

Using a Qiagen kit (USA), total RNA was extracted from the liver, kidney, testis, and brain tissues and evaluated using the RT-PCR method. Then, Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, USA) catalyzed the inverse transcription of the extracted RNA into complementary DNA (cDNA). Real-Time PCR System (Step One Plus) and a SYBR® Green PCR Master Mix (Applied Biosystems) were conducted in a 10 µl final volume, programming the heating cycles: 95°C (10 min), then 40 cycles of 95°C (15 s) and 65 °C (1 min). The sequences of PCR primer pairs of Bax: Forward: 5′-TGG CGA TGA ACT GGA CAA CA-3′; Reverse: 5′-TGC CAC ACG GAA GAA GAC C-3′, BCL-2: Forward: 5′-GGA CAA CAT CGC TCT GTG GA-3′; Reverse: 5′-CAT CCC AGC CTC CGT TAT CC-3′, Cyt C: Forward: 5′-GGG CGA GAG CTA TGT AAT GCA AG-3′, Reverse: 5′-TAC AGC CAA AGC AGC AGC TCA-3′ Caspase- 9: Forward: 5′-ACG TGA ACT TCT GCC CTT CC-3′; Reverse: 5′-GGT CGT TCT TCA CCT CCA CC-3′, Caspase-3: Forward: 5′-GAA CGA ACG GAC CTG TGG A-3′; Reverse: 5′-CAG TCC AGC TCT GTA CCT CG-3′, and the housekeeping reference gene beta-actin (β-actin): Forward: 5′-TGT CAC CAA CTG GGA CGA TA-3′; and Reverse: 5′-AAC ACA GCC TGG ATG GCT AC-3′. ABI Prism sequence detection system software and v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA) were used to calculate the results. The relative ratios of the investigated genes were normalized to β-actin using the comparative threshold cycle method and calculated via the expression $2^{-\Delta\Delta Ct}$ **(Pfaffl, 2001)**.

Assessment of the trace elements levels

Milestone MLS-1200 Mega, High-Performance Microwave Digester Unit (Italy) was used to digest the liver, kidney, testis, or brain tissues in a mixture of concentrated nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) (5:1 v/v). An ICP (OES, Perkin Elmer, Optima 2000 DV) was used to determine the levels of the trace elements: calcium (Ca^{2+}), iron (Fe^{3+}), copper (Cu^{2+}), and zinc (Zn^{2+}) in the prepared tissue samples.

Data Examination

The Statistical Package for the Social Sciences (SPSS, Version 23.0) was used to analyze the data. The findings were presented as mean ± standard error (SE). Values were compared by one-way analysis of variance (ANOVA). For intergroup comparisons, post hoc analysis was carried out using the least significant difference (LSD) test. A *p* value of less than 0.05 was deemed statistically significant, and a *p* value less than 0.01 was deemed extremely significant.

RESULTS

Histopathological investigation

The histological investigations of liver slides demonstrated that the liver tissues of C, FSO, and FO groups showed average portal tracts with average portal veins, average hepatocytes in the peri-portal area, average central veins with average hepatocytes arranged in single-cell cords with average intervening blood sinusoids (Figures 1: La, Lb, and Lc). The liver tissues of R group showed mildly edematous portal tracts with markedly dilated congested portal veins and scattered apoptotic hepatocytes in peri-portal area, and markedly dilated central veins with marked hydropic change and scattered apoptosis of hepatocytes in peri-venular area with marked areas of hemorrhage (Figure 1: Ld). The liver tissues of 2,4-D treated group showed mildly edematous portal tracts with mildly dilated congested portal veins and a mild hydropic change of hepatocytes in peri-portal area, and markedly dilated congested central veins with mild hydropic change of hepatocytes in the peri-venular area (Figure 1: Le). The liver tissues of 2,4-D/R group showed mildly edematous portal tracts with mildly dilated portal veins and scattered apoptotic hepatocytes in peri-portal area, and mildly dilated central veins with detached lining, mildly congested blood sinusoids with marked areas of hemorrhage, mild inflammatory infiltrate and scattered apoptosis and mild hydropic change of hepatocytes in the peri-venular area (Figure 1: Lf). The liver tissues of FSO/2,4-D/R group showed mildly edematous portal tracts with mildly congested portal veins and scattered apoptotic hepatocytes in the peri-portal area, and average central veins with mildly congested blood sinusoids blood vessels and scattered apoptotic hepatocytes in the peri-venular area (Figure 1: Lg). The liver tissues of FO/2,4-D/R group showed mildly edematous portal tracts with small areas of hemorrhage, mildly congested portal veins and scattered apoptotic hepatocytes in the peri-portal area, and mildly congested central veins with marked areas of hemorrhage, and scattered apoptosis with marked hydropic change of hepatocytes in the peri-venular area (Figure 1: Lh).

Moreover, the histological investigations of kidney slides showed that: the kidneys of C, FSO, and FO groups showed average renal capsule, average glomeruli with average Bowman's spaces, average proximal tubules with preserved brush borders, average distal tubules, and renal medulla showed average collecting tubules with average peri-tubular capillaries (Figure 1: Ka, Kb, and Kc). The kidney tissues of R group showed average renal capsule, markedly atrophied glomeruli with widened Bowman's spaces, proximal tubules with scattered apoptotic epithelial lining and loss of brush borders, average distal tubules, and renal medulla showed average collecting tubules with average peri-tubular capillaries (Figure 1: Kd). The kidney tissues of 2,4-D group showed average renal capsule, markedly atrophied glomeruli with widened Bowman's spaces, proximal tubules with scattered apoptotic and mildly edematous epithelial liming with loss of

brush borders, and renal medulla showed average collecting tubules with mildly congested peri-tubular capillaries (Figure 1: Ke). The kidney tissues of 2,4-D/R group showed average renal capsule, atrophied glomeruli with widened Bowman's spaces, proximal tubules with scattered apoptotic and mildly edematous epithelial lining, and renal medulla showed average collecting tubules with mildly congested peri-tubular capillaries (Figure 1: Kf). The kidney tissues of FSO/2,4-D/R group showed average renal capsule, average glomeruli with average Bowman's spaces, proximal tubules with average epithelial liming and average interstitial blood vessels, and renal medulla showed collecting tubules with scattered apoptotic epithelial liming and average peri-tubular capillaries (Figure 1: Kg). The kidney tissues of FO/2,4-D/R group showed average renal capsule, small-sized glomeruli with widened Bowman's spaces, proximal tubules with scattered apoptotic epithelial liming and mildly congested interstitial blood vessels, and renal medulla showed collecting tubules with scattered apoptotic epithelial liming and average interstitium (Figure 1: Kd).

However, the histological investigations of testes slides showed that the testis of C, FSO and FO groups showed average tunica albuginea, average-sized tubules with average germinal lining and complete spermatogenesis, average basement membrane, and average interstitium with average Leydig cells (Figure 1: Ta, Tb, and Tc). The testis of R group showed thick tunica albuginea with mildly congested sub-capsular blood vessels, scattered small-sized tubules with atrophied/necrotic germinal lining and spermatogonia only, thick destructed basement membrane, and mild sub-capsular and interstitial edema with average Leydig cells (Figure 1: Td). The testis of 2,4-D group showed thick tunica albuginea with markedly congested and thrombosed sub-capsular and interstitial blood vessels, scattered markedly distorted small-sized tubules with markedly atrophied germinal lining, some tubules lined by spermatogonia only, others with scattered primary spermatocytes and others with markedly apoptotic germinal lining with no spermatogenesis, average basement membrane, and average interstitium with average Leydig cells (Figure 1: Te). The testis of 2,4-D/R group showed thick tunica albuginea with mildly congested sub-capsular blood vessels, most of tubules are small-sized and distorted with atrophied germinal lining, some tubules lined by spermatogonia only and others with scattered primary spermatocytes and no spermatogenesis, thick basement membrane, and mildly edematous interstitium with average Leydig cells (Figure 1: Tf). The testis of FSO/2,4-D/R group showed average tunica albuginea with average sub-capsular blood vessels, average-sized tubules with average germinal lining and complete spermatogenesis, average basement membrane, and mildly edematous interstitium with average Leydig cells (Figure 1: Tg). The testis of FO/2,4-D/R group showed average tunica albuginea with mildly congested sub-capsular blood vessels, scattered small-sized tubules with thin germinal lining and complete spermatogenesis, average basement membrane, and average interstitium with average Leydig cells (Figure 1: Th).

On the other hand, the histological investigations of brain slides showed that the brains of C, FSO, and FO groups showed average meninges with average sub-meningeal blood vessels, average cerebral cortex with average neurons, average glial cells, and average intra-cerebral blood vessels, and the striatum showed average neurons, average glial cells, and average blood vessels (Figure 2: Ba, Bb, and Bc). The hippocampus of C, FSO, and FO groups showed average Cornu Amonis CA1, CA2, CA3, average dentate gyrus (DG), average pyramidal neurons, average inter-neuron area, and average blood vessels (Figure 2: Ha, Hb, and Hc). The brain of R group showed average meninges with mildly congested sub-meningeal blood vessels, the cerebral cortex showed markedly degenerated neurons, average glial cells, and mildly congested intra-cerebral blood vessels with areas of hemorrhage, and the striatum showed scattered degenerated neurons, average glial cells, and eosinophilic plaque-like areas (Figure 2: Bd). The

hippocampus showed markedly degenerated pyramidal neurons in CA1, CA2, CA3, and in DG, eosinophilic plaque area in CA1, and mildly congested blood vessels in (CA1) and in (DG) (Figure 2: Hd). The brain of 2,4-D group showed average meninges with average sub-meningeal blood vessels, cerebral cortex showed markedly degenerated neurons, average glial cells and average intra-cerebral blood vessels, and striatum showed markedly degenerated neurons, average glial cells, and average blood vessels (Figure 2: Be). The hippocampus showed markedly degenerated pyramidal neurons in CA1, CA2, CA3, average DG, average inter-neuron area, and average blood vessels (Figure 2: He). The brain of 2,4-D/R group showed average meninges with mildly congested sub-meningeal blood vessels, the cerebral cortex showed scattered degenerated neurons, average glial cells, average intra-cerebral blood vessels, and eosinophilic plaque-like areas, and the striatum showed average neurons, average glial cells, and mildly congested blood vessels (Figure 2: Bf). The hippocampus showed markedly degenerated pyramidal neurons in CA3, scattered degenerated pyramidal neurons in CA1, CA2, and in the DG, eosinophilic plaque-like areas and mildly congested blood vessels in the DG (Figure 2: Hf). The brain of FSO/2,4-D/R group showed average meninges, cerebral cortex showed scattered degenerated neurons, average glial cells, and mildly congested intra-cerebral blood vessels, and the striatum showed average neurons, average glial cells, and average blood vessels (Figure 2: Bg). The hippocampus showed average Cornu Amonis (CA1), (CA2), (CA3), average DG, average pyramidal neurons, average inter-neuron area, and average blood vessels (Figure 2: Hg). The brain of FO/2,4-D/R group showed average meninges, cerebral cortex showed scattered degenerated neurons, average glial cells, and mildly congested intra-cerebral blood vessels, and the striatum showed scattered degenerated neurons, average glial cells, and average blood vessels (Figure 2: Bh). The hippocampus showed scattered degenerated pyramidal neurons in the CA1, CA2, CA3, eosinophilic plaque-like areas in CA3, and mildly congested blood vessels in CA1 and in the DG (Figure 2: Hh).

Tables 1-5 represent the histopathological scoring of the liver, kidney, testis, brain cortex and striatum, and brain hippocampus, respectively, in normal and 2,4-D/R treated rats with FSO and FO oils.

Table 1: Histopathological scoring of the liver in normal and 2,4-dichlorophenoxy acetic acid/gamma-irradiation treated rats with flaxseed and fish oils:

Group	Portal tract				Peri-venular area				
	PV	Inflammatory infiltrate	Edema	Hepatocytes	CV	Blood sinusoids	Hepatocytes	Inflammat infiltrate	Hge
Control	0	0	0	0	0	0	0	0	0
FSO	0	0	0	0	+	0	0	0	0
FO	+	0	+	0	+	0	0	0	+
R	++	0	+	+	++	0	+	0	++
2,4-D	+	0	+	+	++	0	+	0	0
2,4-D/R	+	0	+	+	+	+	+	+	++
FSO/2,4-D/R	+	0	+	+	0	+	+	0	0
FO2,4-D/R	+	0	0	+	+		+	0	++

- **Portal tract:**
  - **Portal vein (PV):** 0**:** Average, **+:** Mildly dilated/congested, **++:** Markedly dilated/congested.
  - **Inflammatory infiltrate:** 0**:** No, **+:** Mild, **++:** Moderate/marked.
  - **Edema:** 0**:** No, **+:** Mild, **++:** Moderate/marked
  - **Hepatocytes:** 0**:** Average, **+:** scattered apoptosis/mild hydropic change, **++:** Marked apoptosis.
  - **Peri-venular area:**
  - **Central vein:** 0**:** Average, **+:** Mildly dilated/congested, **++:** Markedly dilated/congested
  - **Blood sinusoids:** 0**:** Average, **+:** Mildly dilated/congested, **++:** Markedly dilated/congested.
  - **Hepatocytes:** 0**:** Average, **+:** scattered apoptosis/mild hydropic change, **++:** Marked apoptosis.
  - **Hemorrhage:** 0**:** No, **+:** Mild, **++:** Moderate/marked.

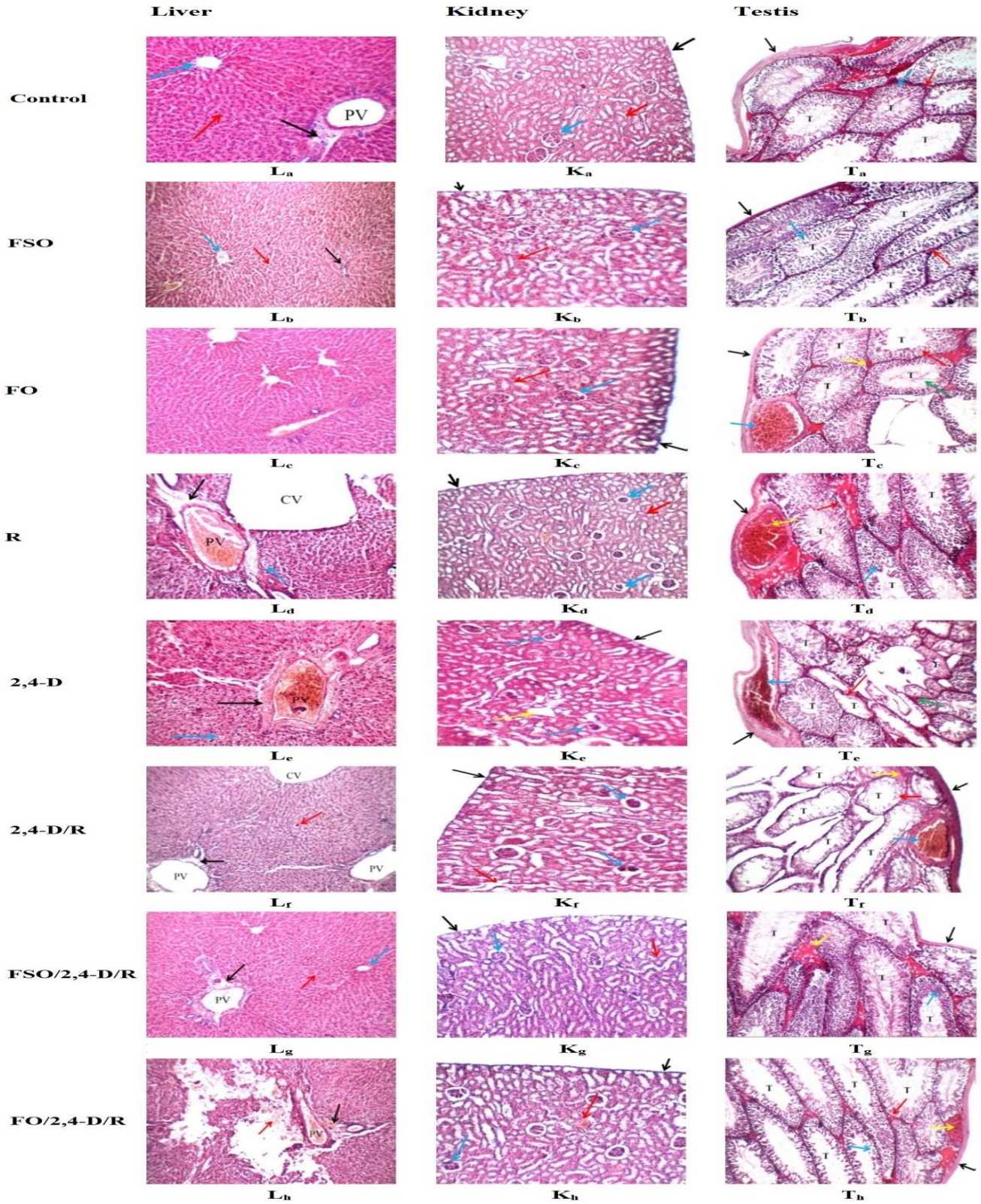

Figure 1. Histopathological investigation of liver, kidney, and testis tissues of different treated groups (H&E X 200).

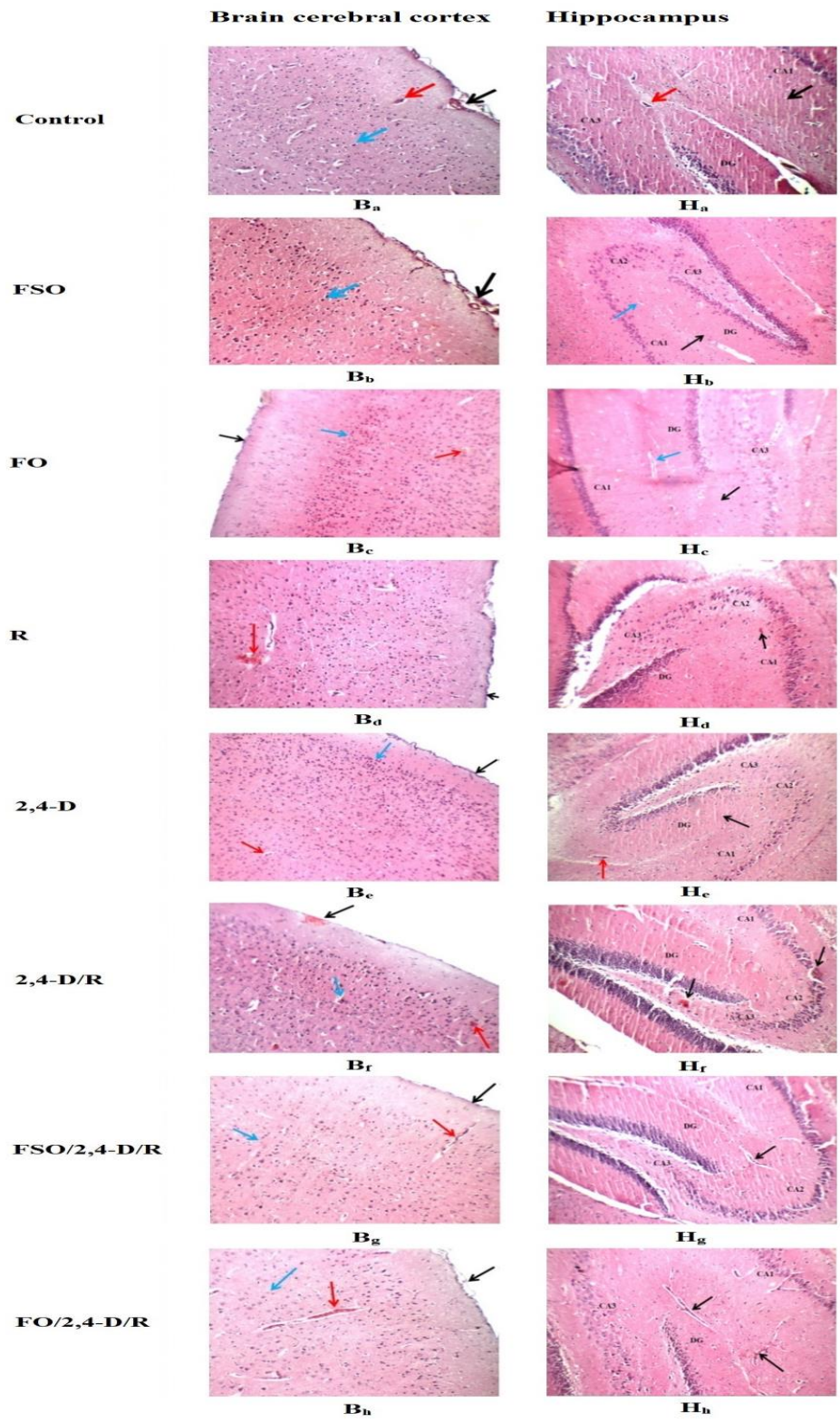

Figure 2. Histopathological investigation of brain tissues of different treated groups (H&E X 200).

Table 2: Histopathological scoring of the kidney in normal and 2,4-dichlorophenoxy acetic acid/gamma-irradiation treated rats with flaxseed and fish oils:

Group	G	BS	Tubules			Interstitium	Medulla
			Lining	Brush border	Lumen		
Control	0	0	0	0	0	0	0
FSO	0	0	0	0	0	0	0
FO	0	0	+	0	0	0	0
R	++	+	+	++	0	0	0
2,4-D	++	+	+	++	0	0	+
2,4-D/R	++	+	+	0	0	0	+
FSO/2,4-D/R	0	0	0	0	0	0	+
FO2,4-D/R	++	+	+	0	0	+	+

- # Glomeruli (G): 0: Average, +: Edematous/congested, ++: Small-sized/atrophied,
- # Bowman's spaces (BS): 0: Average, +: Widened/dilated, ++: Obliterated
- # Tubules Lining: 0: Average, +: Edematous/apoptotic, ++: Atrophied/necrotic
- Brush border: 0: Preserved+: Partial loss++: Complete loss
- Lumen: 0: Free+: Few/scattered casts++: Marked Intra-tubular casts
- # Interstitium: 0: Average, +: Dilated/congested BV, ++: Markedly dilated BV/interstitial hemorrhage
- # Medulla: 0: Average, +: Congested capillaries/scattered hyaline casts, ++: Marked hyaline casts.

Table 3: Histopathological scoring of the testis in normal and 2,4-dichlorophenoxy acetic acid/gamma-irradiation treated rats with flaxseed and fish oils:

Group	Tunica albuginea	BV	Tubules	Germinal lining	Spermatogenesis	BM	Interstitium
Control	0	0	0	0	0	0	0
FSO	0	0	0	0	0	0	0
FO	0	+	0	0	0	0	0
R	+	+	+	++	+	++	+
2,4-D	+	++	++	++	++	0	0
2,4-D/R	+	+	++	++	++	+	+
FSO/2,4-D/R	0	0	0	0	0	0	+
FO2,4-D/R	0	+	+	+	0	0	0

- **Tunica albuginea: 0**: Average, **+:** Thick, **++:** Thick/edematous
- **BV: 0**: Average, **+:** mildly congested, **++:** Markedly congested/thrombosed
- **Tubules: 0:** Average size, **+:** Small-sized in less than 50%, **++:** small-sized or distorted in more than 50%
- **Germinal lining: 0:** Average, **+:** Thin, ++: Apoptotic/ atrophied
- **Spermatogenesis: 0:** Complete **+:** Focal**++:** No spermatogenesis
- **Basement membrane (BM): 0:** Average, BM**+:** Thick, BM**++:** Thick/destructed
- **Interstitium: 0:** Average, interstitium**+:** Mildly edematous, **++:** Markedly edematous/apoptotic cells

Table 4: Histopathological scoring of the brain Cortex and Striatum in normal and 2,4-dichlorophenoxy acetic acid/gamma-irradiation treated rats with flaxseed and fish oils:

Group	Cortex					Striatum			
	Meninges	Neurons	Glial cells	BV	Background	Neurons	Glial cells	BV	Background
Control	0	0	0	0	0	0	0	0	0
FSO	0	0	0	0	0	0	0	0	0
FO	0	0	0	0	0	0	0	0	0
R	0	++	0	+	++	+	0	0	+
2,4-D	0	++	0	0	0	++	0	0	0
2,4-D/R	0	+	0	+	+	0	0	+	0
FSO/2,4-D/R	0	+	0	+	0	0	0	0	0
FO2,4-D/R	0	+	0	+	0	+	0	0	0

- **Meninges: 0:** Average, **+:** Detached, **++:** Markedly thickened
- **Neurons: 0:** Average, **+:** Scattered degenerated, **++:** Markedly degenerated
- **Glial cells: 0:** Average, **+:** Scattered degenerated, **++:** Markedly degenerated
- **BV: 0:** Average, **+:** Dilated/congested, **++:** Markedly dilated
- **Background: 0:** Average, **+:** Eosinophilic plaque-like areas, **++:** Areas of necrosis/Hge/calcification

Table 5: Histopathological scoring of the brain Hippocampus in normal and 22,4-dichlorophenoxy acetic acid/gamma-irradiation treated rats with flaxseed and fish oils:

Group	Hippocampus					
	CA1	CA2	CA3	DG	Inter-neuron area	BV
Control	0	0	0	0	0	0
FSO	0	0	0	0	0	0
FO	0	0	0	0	0	0
R	++	++	++	++	++ In CA1	+ In CA1, DG
2,4-D	++	++	++	0	0	0
2,4-D/R	+	+	++	+	++ In DG	+ In DG
FSO/2,4-D/R	0	0	0	0	0	0
FO2,4-D/R	+	+	+	+	++ In CA3	+ In CA1, DG

- **Pyramidal neurons: 0:** Average, **+:** Scattered degenerated, **++:** Markedly degenerated
- **Inter-neuron area: 0:** Average, **+:** Edema/hemorrhage, **++:** Eosinophilic plaque-like areas
- **BV: 0:** Average, **+:** Dilated/congested, **++:** Markedly dilated

Assessment of biochemical parameters

The data demonstrated that the activity of the serum liver enzymes (ALT, AST, ALP, and LDH) were highly significantly elevated ($p < 0.01$). Moreover, the levels of urea, creat, TC, and TG were highly significantly enhanced ($p < 0.01$), however, the TP, and ALB levels were significantly declined ($p < 0.01$) in the R, and 2,4-D, as compared to the corresponding levels. These values were boosted in the 2,4-D/R intoxicated animals (Figure 3). However, FSO and FO significantly ameliorated these biochemical parameters in the 2,4-D/R intoxicated animals towards their control values (Figure 3).

Sperm Analysis: Sperm count, and motility

The data demonstrated that the sperm count demonstrated highly significantly elevation ($p \leq 0.01$) in the FSO and FO-treated groups by 1.3 and 1.4 folds. However, the sperm count established highly significant declines ($p < 0.01$) in the R, and 2,4-D treated groups. The sperm count showed high close down ($p < 0.01$) in the 2,4-D/R intoxicated groups, as compared to the control group' sperm count. The sperm motility showed also high significant declines in the R, 2,4-D, and 2,4-D/R intoxicated groups. However, FSO and FO treatments recovered the sperm count, and ameliorated the sperm motility in the 2,4-D/R intoxicated animals (Figure 4).

Reproductive Hormonal changes in serum

The data showed that the TH levels showed high significant declines ($p < 0.01$). However, the levels of FSH, and LH established high significant increases ($p < 0.01$) in the serum of R, 2,4-D, and 2,4-D/R intoxicated rats, in contrast to the control levels. FSO and FO improved the levels of FSH, LH, and T in the serum of 2,4-D/R intoxicated animals (Figure 5).

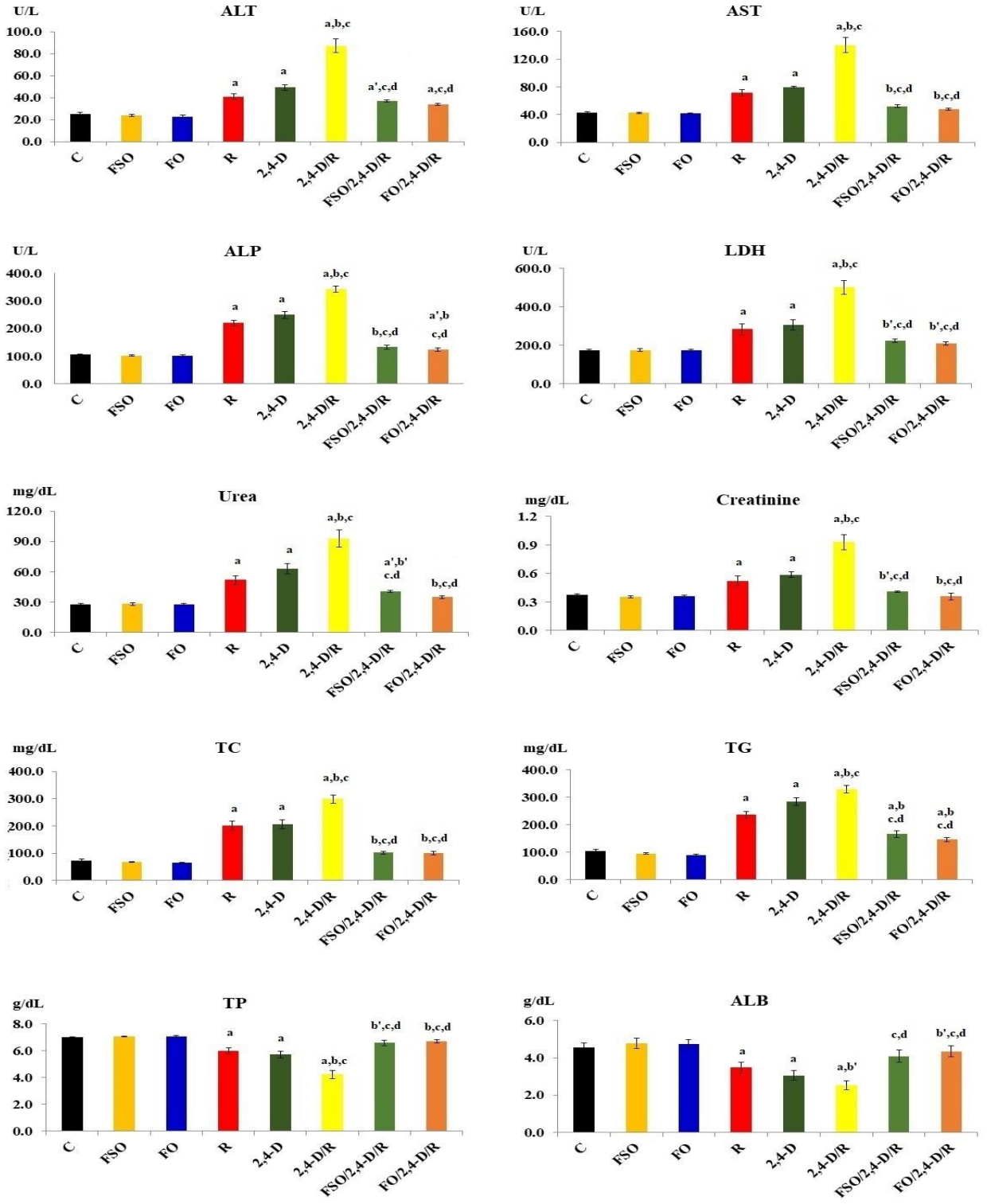

Figure 3. Biochemical parameters in serum.

The statistical significances to C, R, 2,4-D, and 2,4-D/R are denoted by a, b, c, and d, respectively; indicating highly significances at $p < 0.01$, while a', and b' are the significance to the C, and R, respectively, at $p < 0.05$. The results are expressed as Mean ± SE (n = 10).

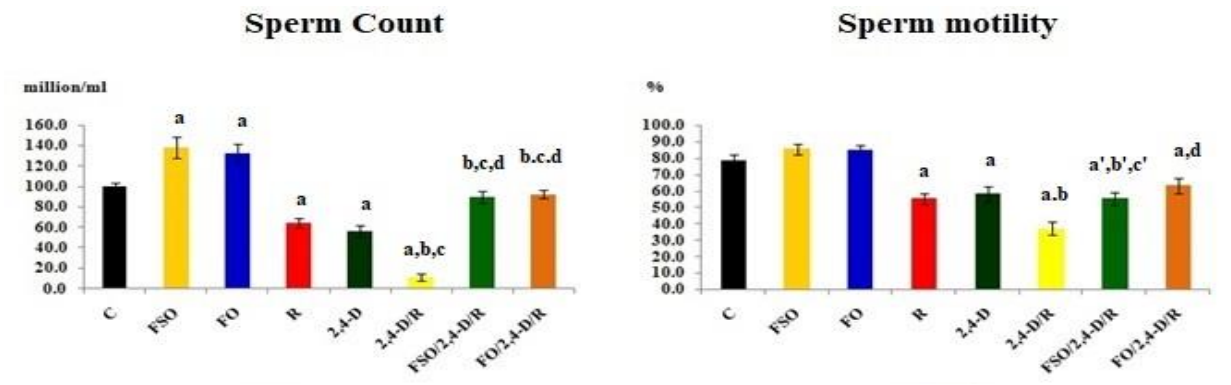

Figure 4. Sperm Analysis: Sperm count, and motility.

a', b', and c' are the significance to the C, R, and 2,4-D, respectively, at $p < 0.05$. Legend as Figure 3.

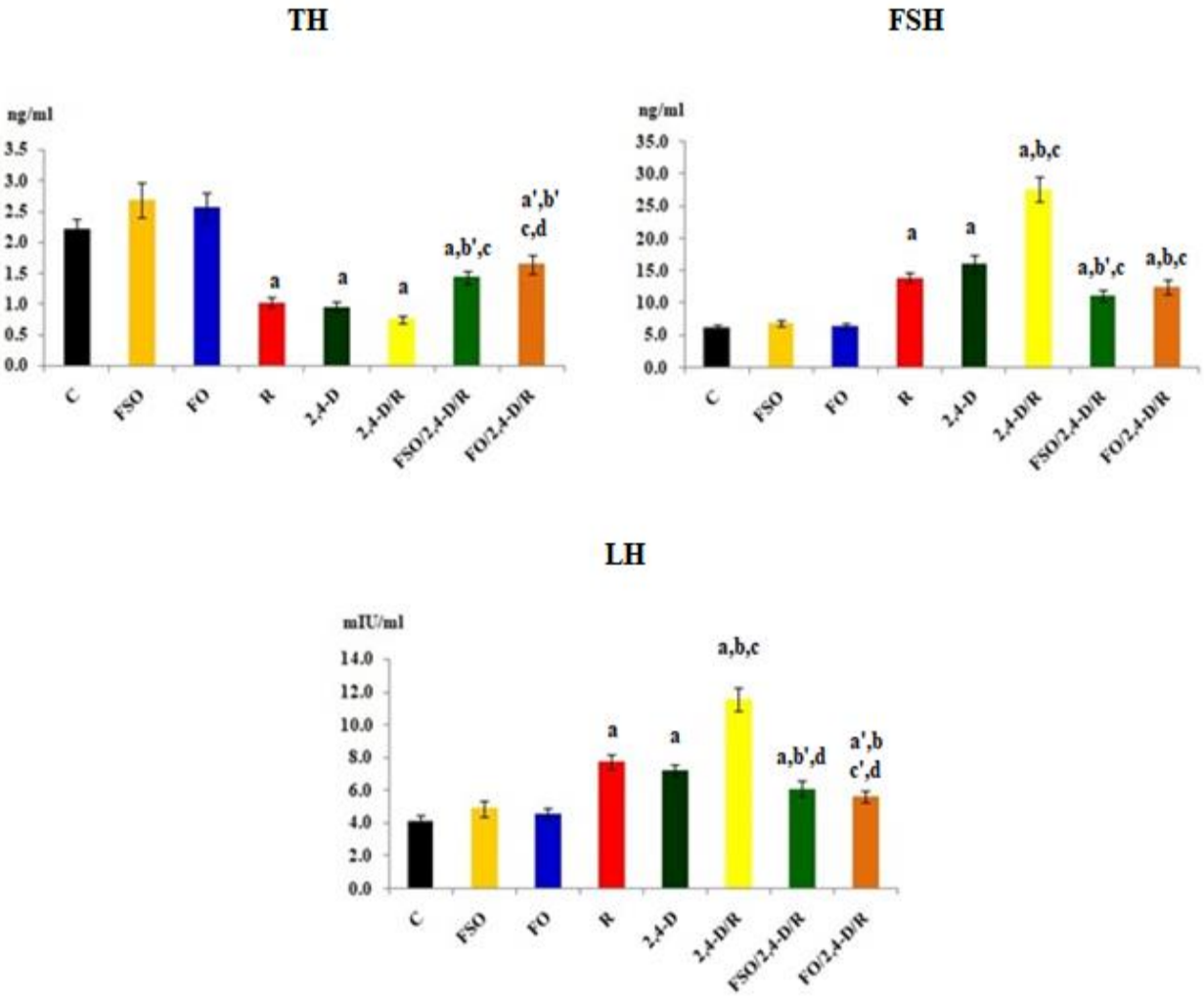

Figure 5. Reproductive hormonal changes in serum.

a', b', and c' are the significance to the C, R, and 2,4-D, respectively, at $p < 0.05$. Legend as Figure 3.

Acetylcholinesterase and Butyrylcholinesterase levels

The data showed that the activities of AchE and BchE were highly significantly inhibited ($p < 0.01$) in the serum of R, 2,4-D, and 2,4-D/R intoxicated rats, correlated to the controls. FSO and FO stimulated the activities of AchE and BchE in the serum of 2,4-D/R intoxicated animals (Figure 6).

Brain dopamine level

The data showed that the levels of dopamine (DA) demonstrated high significant decreases in brain tissues of R, 2,4-D, and 2,4-D/R intoxicated rats, respectively, as compared to the control levels. However, FSO and FO readjusted the levels of DA in the brain tissues of 2,4-D/R intoxicated animals (Figure 6).

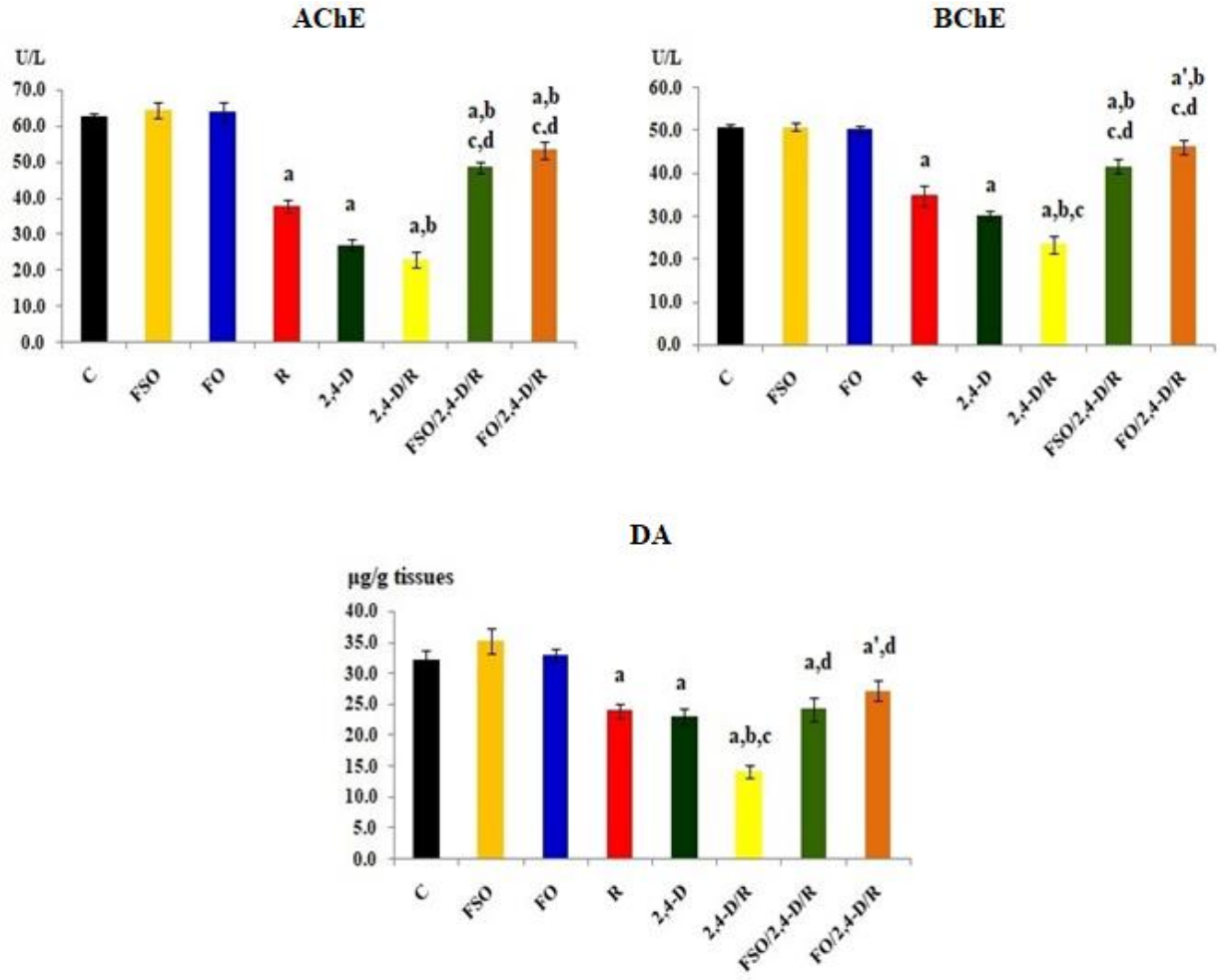

Figure 6. The levels of acetylcholinesterase (AChE), butyrylcholinesterase (BChE) activities in serum, and dopamine level (DA) in the brain tissues.

a' is the significance to the C at $p < 0.05$. Legend as Figure 3.

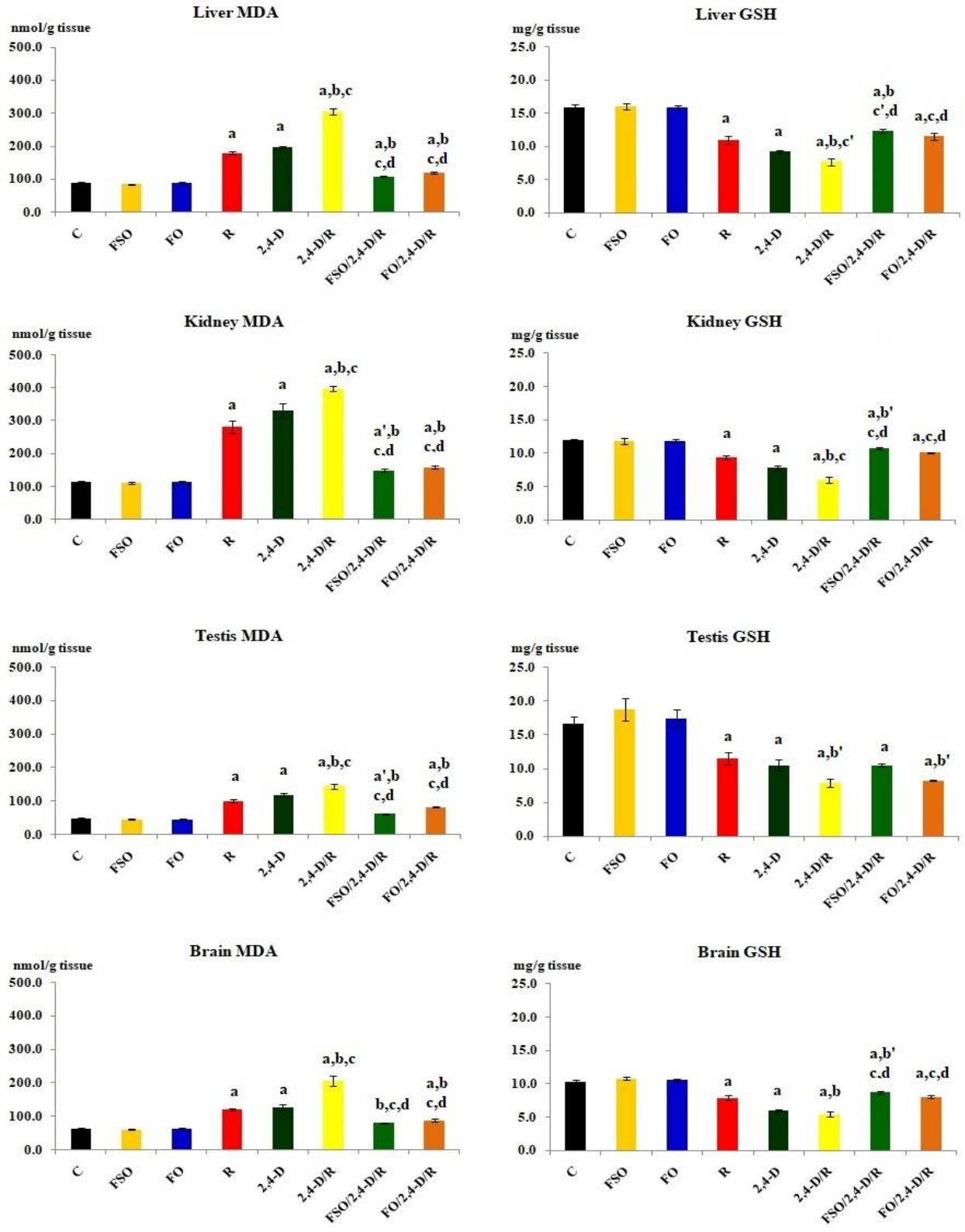

Figure 7. Malondialdehyde (MDA) levels and reduced glutathione (GSH) contents in different tissues.

a', b', and c' are the significance to the C, R, and 2,4-D, respectively, at $p < 0.05$. Legend as Figure 3.

Malondialdehyde levels and glutathione reduced contents

The data showed that the levels of MDA revealed high significant elevations ($p < 0.01$), while, the GSH contents were highly significantly declines ($p < 0.01$) in the liver, kidney, testis, and brain tissues of R, 2,4-D, and 2,4-D/R spoiled rats, corelated to the control levels. However, FSO and FO regulated the MDA levels and GSH contents in different tissues of 2,4-D/R intoxicated animals (Figure 7).

The levels of the inflammatory markers

The data showed that the levels of IL-1β, IL-6, TNF-α, and NF-κB were highly significantly enhanced ($p < 0.01$) in the serum of R, 2,4-D, and 2,4-D/R intoxicated rats, relative to the controls. FSO and FO significantly reduced their levels in the serum of 2,4-D/R intoxicated animals (Figure 8).

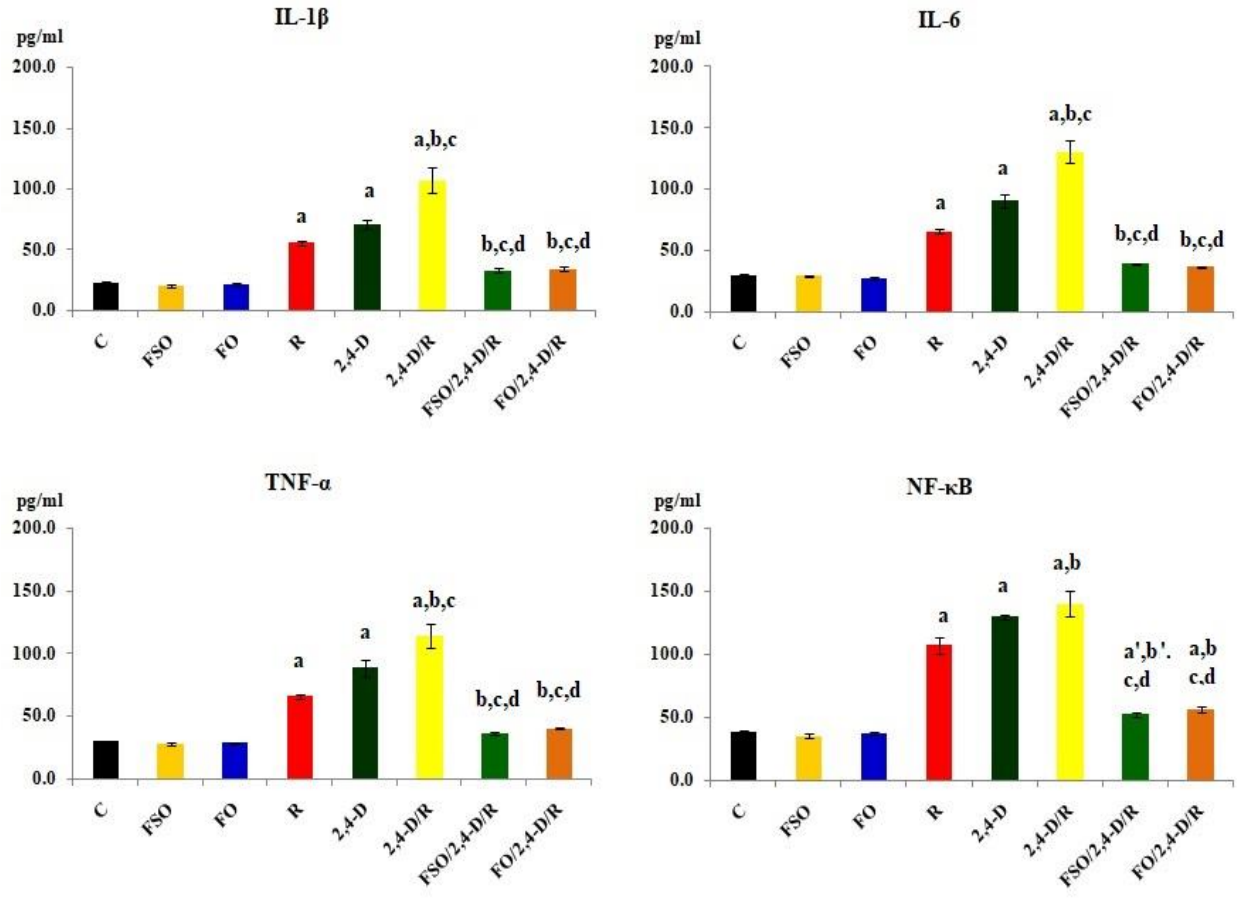

Figure 8. Inflammatory markers level in serum.

a', and b' are the significance to the C, and R, respectively, at $p < 0.05$. Legend as Figure 3.

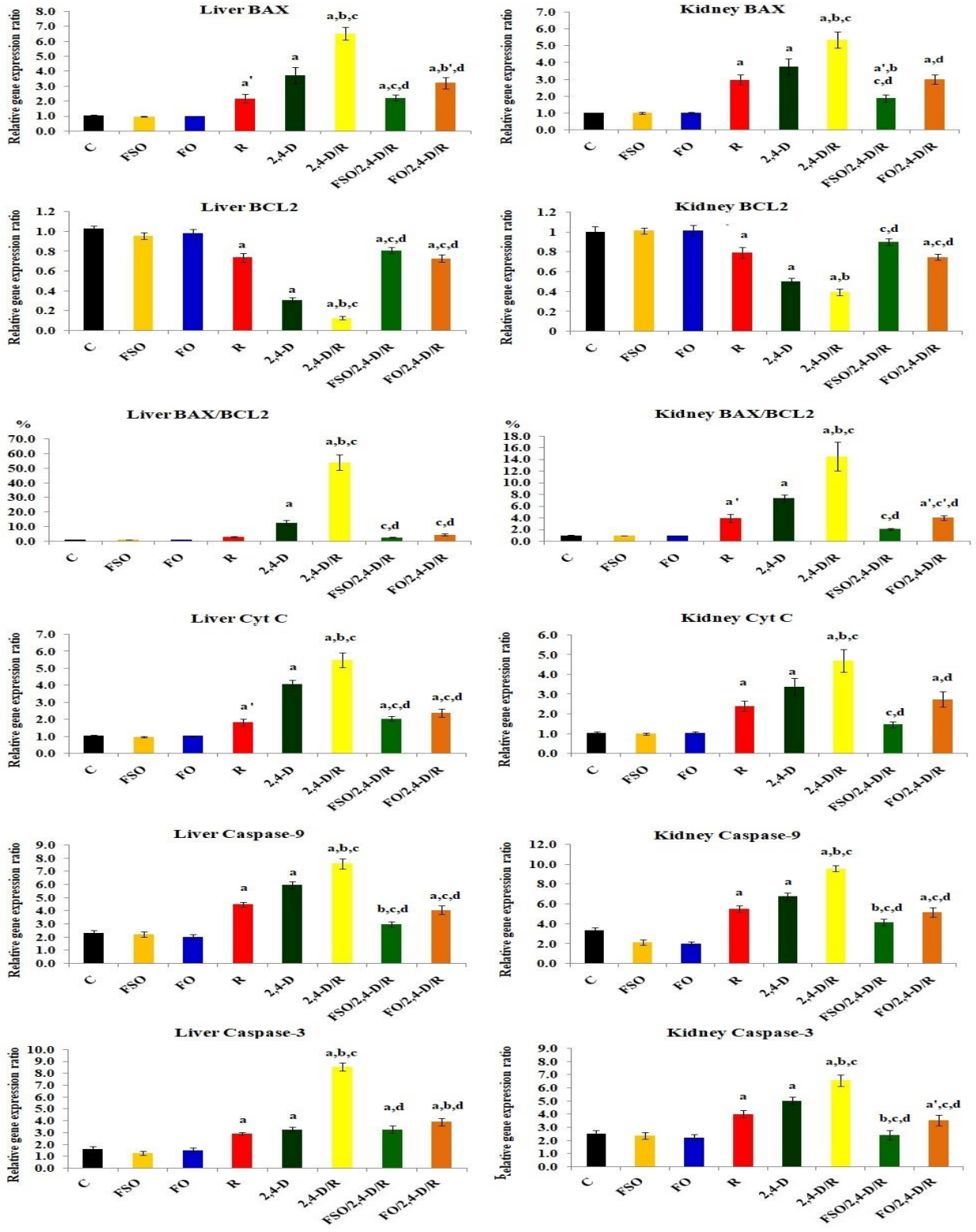

Figure 9: Apoptotic markers in the liver and kidney tissues.

The results are expressed as Mean ± SE (n = 6). a', b' and c' are the significance to the C, R, and 2,4-D, respectively, at $p < 0.05$. Legend as Figure 3.

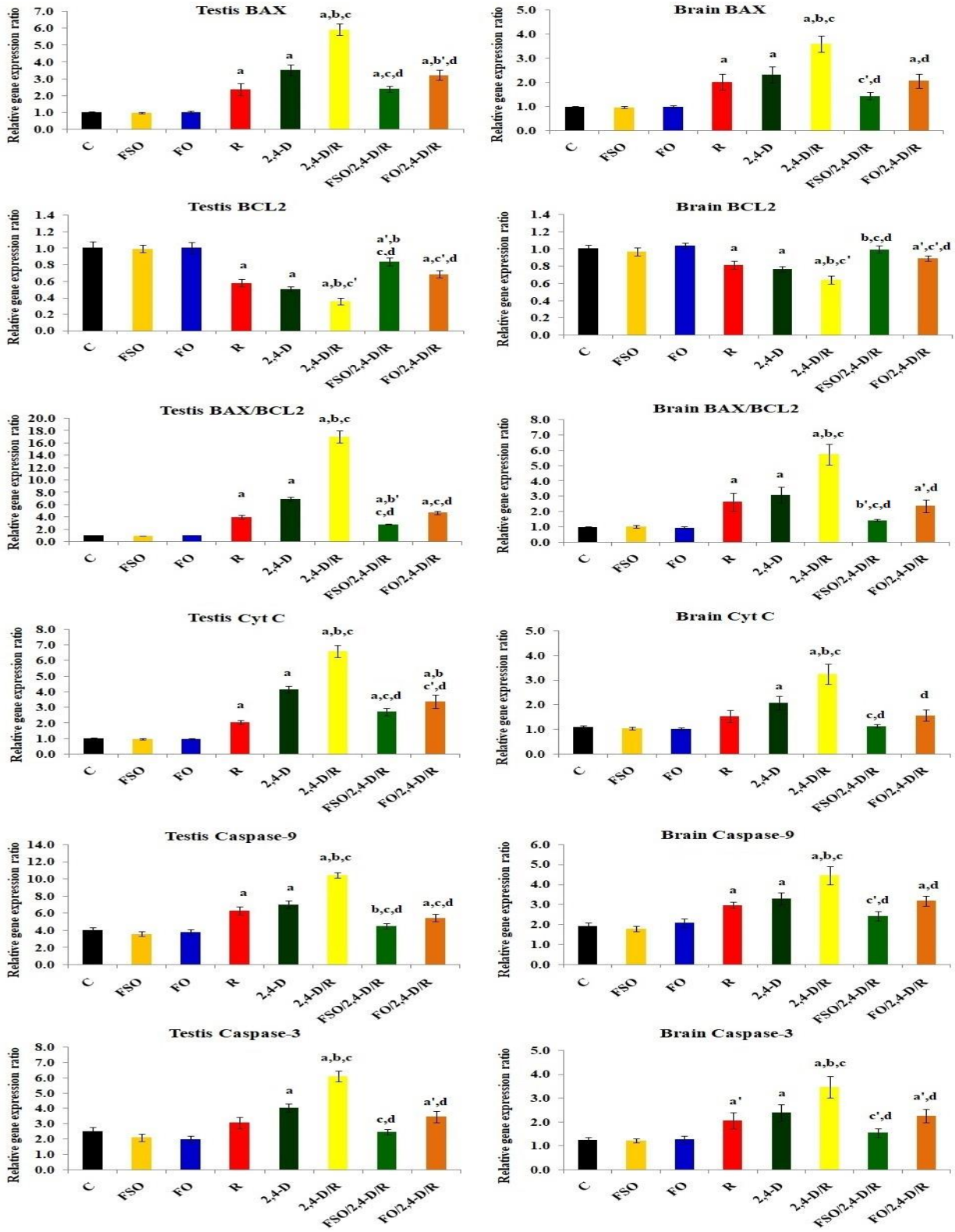

Figure 10: Apoptotic markers in the testis and brain tissues.

The results are expressed as Mean ± SE (n = 6). a', b' and c' are the significance to the C, R, and 2,4-D, respectively, at $p < 0.05$. Legend as Figure 3.

Apoptotic markers in different tissues

The data presented that the gene expression ratios of BAX, Cyt C, caspase-9, and caspase-3 were highly significantly up-regulated ($p < 0.01$), the gene expression ratios of BCL2 showed high significant down-regulation ($p < 0.01$), while BAX/BCL2 ratio were highly significantly increased ($p < 0.01$) in the liver, kidney, testis, and brain tissues of R, 2,4-D, and 2,4-D/R spoiled rats, relative to the controls. FSO and FO managed the gene expression ratios of BAX, BCL2, Cyt C, caspase-9, and caspase-3 and significantly declined BAX/BCL2 ratio in the liver, kidney, testis, and brain tissues of R, 2,4-D, and 2,4-D/R spoiled rats, relative to the controls (Figure 9 and Figure 10).

Trace elements levels in different tissues

The levels of trace elements Ca^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+} showed alteration in the liver, kidney, testis, and brain tissues of the intoxicated groups (Figure 11). The level of Ca^{2+} showed significant elevation ($p < 0.05$) in the liver, and high significant elevations ($p < 0.01$) in the kidney, testis, and brain tissues of R, 2,4-D, and 2,4-D/R treated animals ($p < 0.05$), relative to the control. FSO and FO treatments regulated the levels of Ca^{2+} towards the corresponding control levels in the liver, kidney, testis, and brain tissues.

The level of Fe^{3+} showed significant elevation ($p < 0.05$) in the liver, and high significant elevations ($p < 0.01$) in the kidney, testis, and brain tissues of R, 2,4-D, and 2,4-D/R treated animals, relative to the control group. FSO and FO treatments regulated the levels of Fe^{3+} towards the corresponding control levels in the liver, kidney, testis, and brain tissues.

The level of Cu^{2+} showed high significant declines ($p < 0.01$) in the liver of R, 2,4-D, and 2,4-D/R treated animals, while the level of Cu^{2+} showed high significant elevations ($p < 0.01$) in the kidney, testis, and brain tissues in R, 2,4-D, and 2,4-D/R treated animals, relative to the control group. FSO and FO treatments regulated the level of Cu^{2+} towards the corresponding control values in the liver, kidney, testis, and brain tissues.

The level of Zn^{2+} showed high significant declines ($p < 0.01$) in the liver, testis, and brain tissues of 2,4-D/R treated animals, while it showed a significant elevation ($p < 0.01$) in the kidney tissues in the 2,4-D/R co-toxicity, relative to the control group. Reversely, FSO and FO treatments regulated the levels of Zn^{2+} towards the corresponding control values in the liver, kidney, testis, and brain tissues.

DISCUSSION

In the current study we investigated the protective effects of FSO, or FO against the toxicities of 2,4-D/R in rats. 2,4-D showed toxic effects that boosted by the combination with gamma-irradiation in the 2,4-D/R group. The data demonstrated that 2,4-D provoked toxicity within different organs. Histopathological alterations in different organs confirm these toxic effects. The negative effects of 2,4-D lead to modifications in the liver, kidneys, testicles, and brain tissues' morphology and physiology **(Ferri et al., 2008, Tayeb et al., 2010, & 2012, Trea et al., 2020)**. It is known that oxygen free radicals and LPO lead to morphological changes in different tissues **(Shafeeq and Mahboob, 2020)**, causing MDA to significantly increase, and GSH content to significantly fall in the liver, kidney, testis, and brain tissues.

The biochemical profile of liver is in line with the hepatocytes' histopathological changes. The activities ALT, AST, ALP, and LDH showed high significant stimulation, and the levels of TG and TC were enhanced, while, TP, and ALB levels were reduced. Moreover, the MDA levels were increased, while GSH contents were decreased, indicating the damaging effect of 2,4-D/R through the oxidative stress, and release of ROS in the hepatocytes.

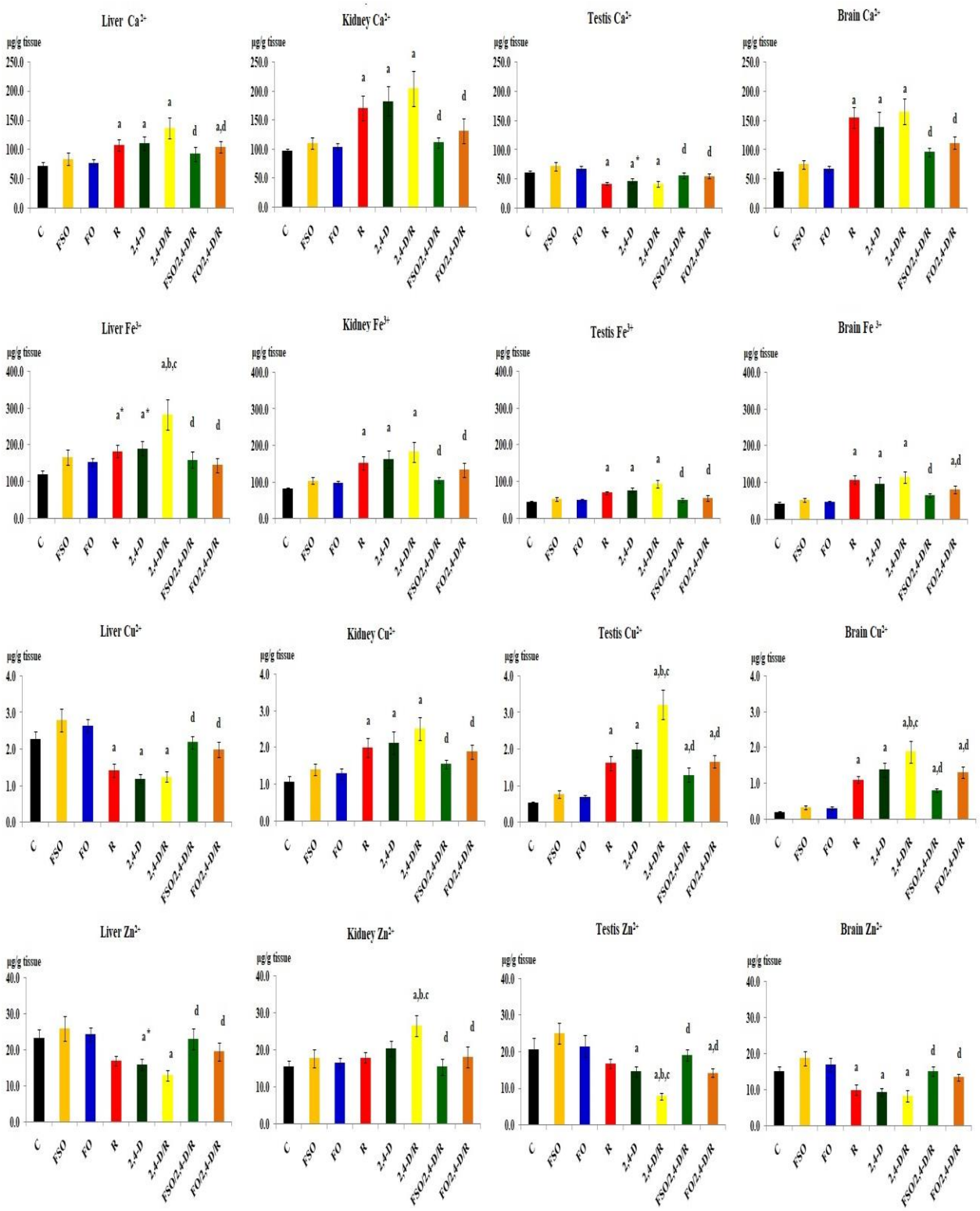

Figure 11. The levels of trace elements in different tissues.

The results are expressed as Mean ± SE (n = 6). Legend as Figure 3.

Furthermore, apoptotic cascade was activated, as the apoptotic molecules BAX, Cyt C, caspase-9, and caspases-3 were up-regulated, while the anti-apoptotic BCL2 expression was down-regulated, resulting in enhancement of BAX/BCL2 ratio in the hepatocytes. This biochemical profile is in line with the histopathological changes in the liver morphology of the 2,4-D/R group that revealed marked areas of hemorrhage, mild inflammatory infiltrate, and scattered apoptosis of the hepatocytes. These results bring out an image of the hepatotoxicity induced in the 2,4-D/R group. This is in line with the previous investigations, in experimental rats treated with 2,4-D **(Tayeb et al., 2010, Shafeeq and Mahboob, 2021, Tichati et al., 2020)**, or exposed to gamma-irradiation-induced hepato-toxicities **(Eassawy et al., 2021, Ismail et al., 2016b, Zaher et al., 2016)**. Alterations in the hepatic enzymes' activities are related to the hepatocytes damage induced by 2,4-D, which modifies the membrane permeability, leading to outflow of the hepatic enzymes to the plasma **(Tichati et al., 2020)**. In addition, the co-toxicity of 2,4-D/R triggered nephrotoxicity, as the kidney morphology developed scattered apoptosis in epithelial lining with other morphological alterations in the renal cells, accompanied by alterations in the urea, and creatinine levels. Renal oxidative stress was confirmed by increasing of MDA level and decreasing GSH contents. Furthermore, apoptotic cascade was activated in the renal cells, with the same form in the hepatocytes, the apoptotic molecules BAX, Cyt C, caspase-9, and caspases-3 were up-regulated, while the anti-apoptotic BCL2 expression was down-regulated, and resulted in enhancement of BAX/BCL2 ratio. Nephrotoxicity was observed after 2,4-D administration **(Shafeeq and Mahboob, 2021, Tayeb et al., 2012, Trea et al., 2020)**, or due to gamma-radiation exposure **(Ismail et al., 2016d, Ismail et al., 2023, Salem and Ismail, 2021)**. Alterations in the urea, and creatinine levels are indicative of renal dysfunction **(Tayeb et al., 2012)**. 2,4-D is accumulated 2,4-D in the renal tissues causing ROS release, which in turn causes severe nephrotoxic effects. This nephrotoxicity is mediated by alterations in the nephrons, tubular and glomerular cells, which cause a drop in the glomerular filtration. The released ROS targets the proteins, and polyunsaturated fatty acids, as well as, triggers oxidative damage, necrosis, and degeneration in the renal tissues **(Tayeb et al., 2012, Trea et al., 2020)**.

In the same context, the morphological changes were observed in the testis, as a scattered primary spermatocyte without spermatogenesis, and some tubules lined by spermatogonia only. These morphological changes can explain the diminished sperm count, alterations in the TH, FSH, and LH levels in the serum, increase of MDA level, and decrease of GSH contents, and the apoptotic cascade that were triggered in the testicular tissues of 2,4-D/R group. 2,4-D administration **(Gabraut and Philbert, 2002, Marouani et al., 2017, Zhang et al., 2017)**, and gamma-radiation exposure **(Gawish et al., 2019)** that induce testicular toxicity. 2,4-D interferes with the hormonal system and triggers endocrine disrupting effects **(Kim et al., 2005)**. 2,4-D endorse hypogonadism, whereas, the gonads produce un-adequate amounts of sex hormones **(Irwig, 2014)**, leading to sexual dysfunction. Exposure to chlorophenoxy herbicides triggers reproductive defects, and drives down the sperm count and motility 5, with abnormal sperms **(Lerda and Rizzi, 1991)**, causing higher rates of birth defects **(Garry et al. 1996)**. Spermatogenesis malformations continue for several months after exposure to chlorophenoxy herbicides **(Jote, 2019, Lerda and Rizzi, 1991)**. 2,4-D exposure leads to poor semen quality, according to the 2,4-D absorbed concentration **(Swan et al., 2003)**. The depressions of sperm counts are related to low fertility **(Raji et al., 2003)**.

Moreover, 2,4-D/R induced neurotoxicity, which is in line with the former investigations of 2,4-D **(Gabraut and Philbert, 2002, Ferri et al., 2008)**, and gamma-irradiation **(Ismail and El-Sonbaty, 2016, Ismail et al., 2016a)**. Exposure to 2,4-D triggers mental defects **(Ong-Artborirak et al., 2022)**. In the 2,4-D/R animals' group, the brain showed histological alterations, the cerebral cortex showed scattered degenerated neurons, and eosinophilic plaque-

like areas, and the striatum showed congested blood vessels. The hippocampus showed degenerated pyramidal neurons in the CA3, CA1, CA2, in the DG, and in the eosinophilic plaque-like areas. Also, DA and GSH contents were dimensioned. In addition, the MDA levels, BAX, Cyt C, caspase-9, and caspases-3 were up-regulated, however, the BCL2 expression was down-regulated, resulting in an increased BAX/BCL2 ratio in the brain tissues of 2,4-D/R co-treated animals. Experiments showed that dopaminergic neurons are influenced by 2,4-D, which can be correlated to a higher incidence of Parkinson's disease **(Tanner et al., 2009)**. The brain contains large and numerous amounts of lipids and fatty acids; thus, it is highly susceptible to oxidative stress that causes damage to the polyunsaturated fatty acids brain contents **(Lee et al., 2020)**. Oxidative stress is generated in different brain areas after 2,4-D administration, producing neurotoxicity mediated by interruption in the blood-brain barrier and in the transport mechanism of the cell membranes **(Bongiovanni et al., 2007, Bjorling-Poulsen et al., 2008, Ferri et al., 2008)**. Moreover, it triggers disturbance in the antioxidant enzymes activity, enhanced the LPO yields, such as MDA, which causes more oxidative stress and damage in brain areas **(Ferri et al., 2008)**.

The data confirmed that the levels of IL-1□, IL-6, TNF-α, and NF-κB were increased in serum in the 2,4-D/R intoxicated rats. **Zhou co-workers (2022)** documented that 2,4-D induced an increase in the IL-1□, IL-6, and TNF-α levels in BV2 microglial cells. In addition, exposure to gamma-irradiation stimulates inflammatory response in different tissues, mediated by activation of pro-inflammatory markers **(Eassawy et al., 2021, Ismail and El-Sonbaty, 2016, Ismail et al., 2016a, Ismail et al., 2016b, Ismail et al., 2023, Zaher et al., 2016)**. 2,4-D and gamma-irradiation activated the programmed cell death or apoptosis induced in various tissues, via activation of caspases-3, leading to DNA fragmentation. Caspases activation is an essential step in apoptosis, and caspases-dependent apoptosis can be reliably detected by measuring caspase-9 activity. Activation of caspase-9 leads to activation of caspases-3, which initiates DNA fragmentation and leads to cell death via mitochondria intrinsic pathway **(Ott et al., 2007, Zhang et al., 2017)**.

The determination of cholinesterases is a significant guide of the pesticide exposure and health drawback correlation. The suppression of cholinesterases activity by pesticides leads to the accumulation of acetylcholine and butyrylcholine, which produce health problems **(Colović et al., 2013)**. The determination of AChE, and BChE activities in serum can be a beneficial marker of the poisoning effects of the pesticides and is considered as a marker of membranes' destruction. 2,4-D administration suppressed the AChE activity in the plasma, brain, and human erythrocytes **(Bukowsk and Hutnik, 2006, Santi et al., 2011, Islam et al., 2018)**. AChE catalyzes the decomposition of acetylcholine (ACh) into choline and acetate in the synaptic cleft. Depression of AChE activity causes accumulation of the ACh in the nerve synapses or neuromuscular junctions, leading to an over-activation in the brain and muscular tissue **(Richardson et al., 2019)**. 2,4-D forms a complex with acetylcholine, acts as false cholinergic messengers, leading to modifications in the neurons, and neurotransmission disturbance **(Sastry et al., 1997, Bjørling-Poulsen et al., 2008)**. The data showed significant inhibitions of AChE and BChE in serum of intoxicated animals.

The levels of trace elements Ca^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+} showed alteration in the liver, kidney, testis, and brain tissues of the intoxicated groups; R, 2,4-D, and 2,4-D/R. Ca^{2+} levels were increased in the liver, kidney, and brain tissues, but decreased in the testis tissues. Fe^{3+} levels were increased in all the investigated organs' tissues of the intoxicated groups; R, 2,4-D, and 2,4-D/R. While, Cu^{2+} levels were increased in the kidney, testis, and brain tissues, and decreased in the liver tissues, as well as, Zn^{2+} levels were decreased in the liver, testis, and brain tissues, but increased in the kidney tissues of 2,4-D/R intoxicated animals. **Shafeeq and Mahboob (2020)**

demonstrated significant increases in Ca^{2+}, Fe^{3+}, and Cu^{2+} levels, but a significant decrease in Zn^{2+} levels in the hepatic tissues, as well as, they reported significant decreases in Ca^{2+}, Fe^{3+}, and Zn^{2+} levels, with a significant increase in Zn^{2+} levels in the kidney tissues of 2,4-D intoxicated rats. In addition, exposure to gamma-irradiation demonstrated trace elements disturbance in different organs **(Eassawy et al., 2021, Ismail and El-Sonbaty, 2016, Ismail et al., 2016a, Ismail et al., 2016b, Ismail et al., 2016d, Ismail et al., 2023, Salem and Ismail, 2021)**. Trace elements have significant biological and metabolic roles, as well as, stabilize the cellular structures in the living cells. Alteration of the trace elements' levels is associated with oxidative stress, apoptosis, and convinced pathological conditions in different organs **(Shafeeq and Mahboob, 2020)**. Ca^{2+} is an essential trace element for the polarization and permeability of biological membranes **(Sharma and Vijayaraghavan, 2001).** Under oxidative stress conditions, Ca^{2+} flows from the extracellular environment, endoplasmic reticulum (ER), and sarcoplasmic reticulum into the cytoplasm, then into the mitochondria and nuclei. Mitochondrial Ca^{2+} involves in the interruption of normal metabolism causing cell death. While, in nuclei, Ca^{2+} modifies the gene transcription and nucleases that are involved in cell apoptosis. Ca^{2+} is involved in the release of Cyt C, therefore triggers caspases-3 activation via the mitochondria pathway, leading to apoptosis **(Ermak and Davies, 2002)**. BAX regulates the apoptotic pathway by stimulating the transportation of Ca^{2+} from ER and enhancing the release of Cyt C **(Nutt et al., 2002)**. Alterations of Cu^{2+}, and Zn^{2+} levels are associated with suppression of SOD activity; especially Cu/Zn-SOD **(Karthikeyan et al., 2007)**, leading to enhancement of LPO accumulation, consuming of GSH, and inhibition of glutathione enzymes system. However, alteration of Fe^{3+} levels is associated with inhibition of catalase, and other Fe^{3+} dependent enzymes in the biological system **(Glorieux and Calderon, 2017)**.

To recover, 2,4-D/R toxicity induced in different tissues, FSO, and FO were administered orally to the intoxicated rats. FSO, and FO treatments have significantly ameliorated the changes in all these studied parameters and reversed the histopathological defects in the investigated tissues, induced by 2,4-D/R toxicity in rats. The data showed that the fatty acid profile in FSO demonstrated high amounts of ALA and LA. However, the fatty acid profile of FO showed high amounts of EPA, followed by DHA. FSO showed a prominent hepato-protective effect on mercuric chloride **(AlRamadneh et al., 2022)**, a nephro-protective effect against sodium arsenate-induced renal injury **(Rizwan et al., 2014)**, FSO ameliorated the hepatic enzymes and kidney function in intoxicated rats **(AlRamadneh et al., 2022, Rizwan et al., 2014)**. In addition, FSO revealed considerable protection against lead acetate-induced testicular oxidative stress **(Abdel Moniem et al., 2010)**, and neuro-protection against gamma-irradiation and carbon tetrachloride-induced brain toxicity **(Ismail et al., 2016a)**. The ALA components of FSO are transformed into DHA and EPA in the body, while LA is metabolized into arachidonic acid. These fatty acids improve the membrane integrity and functions, and speed up the repair mechanism of injured organelles, such as mitochondria and plasma membrane, thus restricting ROS production and enhancing the antioxidant enzymes activity. Moreover, the lignin components of flaxseed oil revealed scavenging activity on the hydroxyl radicals and suppressed LPO **(Diab et al., 2020, Shahidi et al., 2022)**. FSO showed significant effects in lowering TG and TC levels and other serum lipids, due to the suppression of very low-density lipoprotein (VLDL) and TG synthesis in the liver with enhancement of lipase activity in the peripheral tissues, and high-density lipoprotein (HDL) levels in the serum. DHA and lignin phytochemical can demonstrate significant effects in lowering TG and TC levels as well as other serum lipids **(Shahidi et al., 2022)**. Furthermore, FSO administration can raise up the reproduction rate in some animal species. ALA of FSO can improve reproductive health, and fertility rate. ALA and its metabolite EPA improve the synthesis of the reproductive hormones, while, DHA protects the

sperms against oxidative stress, enhances testosterone concentration in the serum, and improves the semen production, spermatogenesis, sperm motility, and testicular function **(Perumal et al., 2023)**. Administering FSO shields brain tissue from severe oxidative damage, due to the powerful anti-inflammatory and antioxidant properties, by blocking the expression of the XO and iNOS genes, depressing NF-κB, and then blocking the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. Moreover, ALA, ω-3 FAs, and lignan phytochemical are responsible for the protective effects of FSO against γ-irradiation and/or CCl_4-induced sever oxidative stress **(Ismail et al., 2016a)**. The phospholipid structures of the cell membrane are affected by unsaturated fatty acids. Accordingly, unsaturated fatty acids can inhibit the production of ROS, discharge of Cyt C, and caspases activation **(Jangale et al., 2013, Taneda et al., 2010)**. FSO reversed the histopathological defects, exhibited anti-inflammatory effects, via regulation of TNF-α, and exhibited anti-apoptotic effects via regulation of p53, caspases-9, and caspases-3 expressions against cadmium toxicity in liver and kidney tissues **(Diab et al., 2020**). ALA, EPA, and DHA that metabolized into eicosanoids, prostaglandin E2 series, and leukotriene B4 are accountable for resistance of the inflammation. EPA regulated caspase-mitochondrial apoptosis, induced via releasing of caspase-3, caspase-9, and cytochrome c **(Diab et al., 2020, Taneda et al., 2010)**.

Fish oil treatment also demonstrated protective effects of 2,4-D/R toxicity. FO had a prominent hepato-protective effect on doxorubicin-induced hepatotoxicity in rats **(Moussa et al., 2020)**, and on isoniazid-rifampin-induced hepatotoxicity **(Basheer et al., 2017)**, nephroprotective effect against cisplatin-induced nephrotoxicity **(Evrenkaya et al., 2000)**, testicular-protection on methotrexate-induced testicular damage **(Ipek et al., 2022)**, and neuro-protection against lead **(Singh et al., 2017)**, and on gamma-irradiation induced neurotoxicity **(Saada et al., 2014)**. DHA-rich fish oil showed protective effects against chronic oxidative stress in rats' brain **(Asari et al., 2022)**. Also, FO exhibited strong antioxidant **(Singh et al., 2017)**, and anti-inflammatory activities. Treatment with FO diminished the levels of NO, TNF-α, IL-1□, and IL-6 which were elevated by carrageenan-induced paw edema in rats **(De Arruda et al., 2017)**. The levels of plasma TG and TC declined after docosapentaenoic acid (DPA) consumption, which controls the synthesis of fatty acids **(Hirako et al., 2023, Kaur et al., 2011)**. **El Mahdy et al. (2023)** established that EPA inhibited acetic acid-induced ulcerative colitis (UC), via deactivation of serum lactate dehydrogenase (LDH), regulated the antioxidant status, and the pro-inflammatory markers levels, including NF-κB and IL-6. FSO regulated BAX and BCL2 expressions **(Manna et al., 2008)**. DHA alleviates the mitochondrial dysfunction induced by high glucose levels in fish **(Shen et al., 2023)**. EPA promotes mitochondrial apoptosis via the regulation of Bax and Bcl-2 **(Manna et al., 2008, Tsuzuki et al., 2007**).

CONCLUSION

Flaxseed and fish oils showed regulatory effects on 2,4-dichlorophenoxyacetic acid and gamma-irradiation co-toxicity induced multi-organs; mainly the liver, kidney, testis, and brain damages, via regulation of mitochondrial apoptotic pathway and the trace elements levels. Flaxseed and fish oils manifested antioxidant, anti-inflammatory, and anti-apoptotic effects. In addition, they regulated serum reproductive hormones levels, acetylcholinesterase, and butyrylcholinesterase activities, as well as, the dopamine level in the brain tissues, and trace elements levels in different tissues. Accordingly, to protect the persons from the toxicity of 2,4-D pesticide, and gamma-radiation, they are recommended to orally administer the dietary oils; flaxseed and fish oils.

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