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Research Article

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Posted Date: February 19th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-218699/v1

Zinc Oxide Nanoparticles improve Testicular Steroidogenesis Machinery Dysfunction In Benzo [α] Pyrene Challenged Rats

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ABSTRACT

Although Zinc oxide nanoparticles (ZnO NPs) in low doses have potentially positive effects on reproduction by their antioxidant effects, the defensive role of Zinc nanomaterials against environmental pollutants that affect male reproduction has not been adequately studied. We designed our study to assess the impact of ZnO NPs towards reproductive dysfunction induced by Benzo[α]Pyrene (B[a]P). Forty-eight mature male rats were randomly distributed into six equal groups: G1; negative control, G2&3- positive control I &II (either 10 or 30 mg ZnO NPs / kg BW); G4. (150 mg Bap / kg BW), G 5 & 6 (Co-administrated B[a]P with different concentrations of ZnO NPs). Oxidative stress biomarkers, semiquantitative real-time PCR for steroidogenic enzymes (CY11A1, StAR, and 3β- HSD), testosterone levels and histopathology in the liver, kidney, and testicles were examined for this investigation. B[a] P treated group showed significant deterioration in all reproductive parameters and induced oxidative stress. Co-administration ZnO NPs eased oxidative stress and effectively increased the expression of CY11A1, StAR, and 3β- HSD and improved histopathological changes in the examined organs. Our results using the selected doses and with Nano particle properties confirm that ZnO NPs have an obvious ameliorative effect against B[a] P.

Keywords: Zinc Oxide Nanoparticles ; Benzo[α]Pyrene ; expression of steroidogenic enzymes ; Oxidative Stress Biomarkers; Histopathology ; Rats.

1. Introduction

Nanoparticles (NPs) are materials with at least one dimension ≤ 100 nm and have a large surface-to-volume ratio. This allows them to possess unique properties that enable them to interact more effectively with biological systems [1,2]. Nowadays, there are increasing interest regarding the impact of these nanoparticles on human and veterinary science, depending on the concern of these nanoparticles easily pass through the blood-brain and blood-testis barrier [3]. ZnO NPs have become one of the most useable metal oxide nanoparticles in biological and animal science applications owing to their exceptional properties of biocompatibility, solubility, economic, and low toxicity [4]. This allows ZnO Nps to mimic biomolecules that regulate cell cycle and cellular homeostasis. But there is a great dispute between reproductive scientists, Are Zinc nanoparticles toxic or could they play a reproductive stimulating role. This big question actually resulted in a conclusion that is the NPs effects depend on many factors like the size, the concentration used, the morphology, the synthesis process, the surface area, the cell type tested and the organism type. Small sizes, higher concentrations, and high frequency of administration doses enhance its toxic

effects [5]. Recently, there have been many new researches of ZnO NPs that using it as a protective effect against reproductive toxicity associated chemotherapy drugs, streptozotocin-induced diabetic and Nicotine [6–9].

Further researches are needed to reveal the defensive effects of Zinc nanomaterials against environmental pollutants that induce male reproductive dysfunction. Therefore, Our study was aimed to investigate the protective effect of ZnO NPs in different concentrations in male rats that underwent Benzo [a]pyrene.

Benzo [a] pyrene (B[a]P) is a polycyclic aromatic hydrocarbon (PAH) and most widespread environmental contaminant, originating from the incomplete burning of fossil fuels, tobacco smoke, diesel consumption, and roasted foods [10]. Data conclusively clinched that, even low-to-moderate exposure to BaP has an endocrine disruptor and deleterious effects on male reproductive system and results in steroidogenic dysfunctions [11–13]. B[a]P increases reactive oxygen species (ROS) production and thus oxidative stress, leads to increased lipid peroxidation and causes male infertility [14,15].

In the current study, we examined the expression of some important steroidogenic enzymes; cholesterol side-chain cleavage enzyme (CYP11A1) (Leydig cell-specific gene), steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase (3 β -HSD) using quantitative Real Time-PCR technique and support our result with scope of oxidative stress biomarkers, serum

level of testosterone, sperm count and finally validated by histopathological examination. Our result may be to add new data on the protectivety of our designed doses of ZnONPs on male fertility.

2. Materials and methods

2.1 Chemicals:

We bought Benzo[α]Pyrene and Zinc oxide nanoparticles from Sigma-Aldrich Chemicals Co., St. Louis, MO, USA. Zinc Oxide Nanoparticles Product code: 544906. They have average particle size <100nm, 10-25 m^2/g specific surface area, They have a formula weight of 81.39 g mol^{-1}, and their quality level is 200. Actual surface area is 15.88 m^2/g using the Brunauer-Emmett-Teller (BET) method [16]. they have high chemical stability electrochemical coupling coefficient and high thermo-mechanical stability at ambient temperatures. We prepared our selected doses of Zinc Oxide Nanoparticles by dissolving them in distilled water.

Animal husbandry and experimental design

We used Forty-eight adult Wistar male rats weighing 200-250 g in this experiment. Rats were housed in stainless steel mesh cages in a naturally lit, ventilated room in the animal unit, National Research Centre, Giza, Egypt. We adjusted the lowest ambient temperature was at 30 ± 2 ◦C and 12h light / 12h dark cycle and fed them with a standard rat diet and water provided *ad libitum*. We put

the animals without treatment for a week to acclimate to the new conditions and then treated for 45 consecutive days.

Protocols and procedures of this experiment were approved by the Institutional Ethics Committee of National Research Centre, Egypt, and the experiments were performed as a per guideline of the National Research Centre Ethical Committee for medical research and in compliance with ARRIVE guidelines.

Rats were allocated randomly (**complete randomization**) into six treatments (n = 8 per treatment). **negative control (NC) group:** a normal healthy untreated animals. **ZnONPs10 and ZnONPs 30 groups:** animals were designed as positive control (PC) were treated in the amount of 1ml with 10 or 30 mg/kg BW/day Zinc nanoparticles . **BaP group:** animals were treated with Benzo [a] pyrene (98% HPLC purity) at doses 150 µg/ kg BW /day. **Bap+ZnONPs10; Bap+ ZnONP30 groups:** animals were Co- administrated B[a]P and ZnO NPs with different concentrations. All the previous chemicals were given daily to rats by oral gavage through oral cannula.

Our ZnO NPs 10 mg/kg/BW dose applied in this experiment was selected according to previously published literature [8], while the dose 30mg/kg/BW according to the published review that clarified the impact of Zn nanoparticles on male (in) fertility [5] and B[a]P dose according to Kang [17].

120 At the end of the experiment, we collected blood samples via sinus orbital
121 puncture using un-heparinized pulled Pasteur pipettes. Subsequently, serum was
122 gathered after centrifugation and store at −20C until the assay Testosterone and
123 oxidative stress biomarkers .

124 **2.3. *Determination of steroidogenesis-related genes using Quantitative Real-***
125 ***Time PCR***

126 *2.3.1. RNA isolation and cDNA synthesis*

127 We dissected Epididymides from rats and froze in liquid nitrogen. Total
128 RNA was extracted after homogenization using the standard TRIzol® reagent
129 extraction method (Invitrogen, USA). We determined RNA concentrations at
130 260/280 nm using an ultraviolet spectrophotometer. Purified RNA was
131 immediately transcribed to Single- strand cDNA using a sample containing 500ng
132 of total RNA following the manufacturer's directions (First Strand cDNA
133 Synthesis Kit (MBI Fermentas, Germany). Reverse transcription (RT) was
134 synthesized at total volume of 25 µl using 0.5 µl poly (dT)18 primer and 13 µl
135 RNA. The reaction was run at 37 ◦C for 90 minutes and ended with a step of
136 denaturation at 70 ◦C for 15 min. Afterward, we preserved the cDNA containing
137 tubes in -20 ◦C.
138

139 *2.3.2. Quantitative Real time-polymerase chain reaction (RT-PCR) using SYBR*
140 *Green I*

141 Real-time RT-PCR analysis for StAR, 3 β -HSD and cholesterol side-chain
142 cleavage enzyme CYP450scc (CYP11A1 gene; Leydig-cell-specific biomarker)
143 were performed on a real-time PCR detection system (iQ5-Bio-Rad Laboratories,
144 Cepheid, USA) using Syber green PCR master mix (TaKaRa, Biotech. Co. Ltd.)
145 The expressions level of our genes mRNAs were normalized to the β -actin
146 housekeeping gene (Actb)**.** We got our target gene and Actb oligonucleotides
147 sequence from the published literatures [18,19] (Table 1). PCR reactions were set up
148 in 25 µl reaction mixtures containing 12.5 µl 1× SYBR, 0.5 µl forward primers,
149 0.5 µl reverse primer, 6.5 µl distilled water, and 5 µl of cDNA template. The
150 amplification cycle started by a preliminary denaturation step at 95 °C for 3
151 minutes followed by 35 cycles of denaturation at 95°C for 15 sec; followed by
152 annealing step at 55°C for 30 sec. Finally, the extension step performed at 72°C
153 for 30 sec. Samples and controls were run in duplicate. Amplification was
154 followed by melt curve analysis to ensure that no primer-dimer amplification
155 occurred. The gene expressions were calculated using the formulae of Bio-Rad
156 laboratories Inc. Ef = 10-1/slope. Efficiency (%) = (Ef - 1) × 100 [20]. We
157 determined The relative quantification of the target to the reference by using the

158 ΔC_T method if E for our target genes and the reference primer (ACTB) are the
159 same Ratio $_{(reference/target)} = Ef^{CT(reference)-CT(target)}$

160 ***2.4. Estimation of serum testosterone and oxidative stress biomarkers :***

161 We determined serum testosterone concentrations using an enzyme-linked
162 immunosorbent assay kit (XEMA Co., LTD, Moscow, Russia) according to the
163 manufacturer's instruction.

164 The level of serum Malondialdehyde (MDA) was estimated
165 colorimetrically using (TBA) thiobarbituric acid method according to the
166 standard Ohkawa method [21].

167 Reduced Glutathione (GSH) activity was based on measurement of the
168 optical density of yellow color derivative coming from GSH and DTNB
169 (nitrobenzoic acid) reaction according to the basic method [22]. We measured The
170 levels of serum MDA and GSH using Bio-diagnostic Co., kits .

171 ***2.5. Determination of sperm count***

172 According to the previously established method [23], we isolated Cauda
173 epididymides and immediately immersed in normal physiological saline, gently
174 shaken for 10 minutes, and then incubated for 2 min at 37 °C to permit
175 spermatozoa to leave the epididymal tubules. A solution of 5 g sodium
176 bicarbonate, 25 mg eosin and 1 ml formalin (35%) dissolved in 100 ml distilled
177 water was prepared and mixed with 1ml supernatant fluid (1:100). An aliquot of

this diluted sperm suspension (10µl) was conveyed to each counting chamber of a hemocytometer, then counted under a light microscope at × 200 magnification.

2.6. Histopathological examination

At the termination of the experiment, we used sodium pentobarbital anesthesia for euthanasia scarification of rats. Testis, liver, and kidney specimens were collected and quickly washed with normal saline then fixed in 10% formalin for histopathological examination. Tissue samples were embedded for 24h in paraffin. We prepared tissue thickness (4-5µm) from paraffin beeswax blocks using a sledge microtome and stained them with Hematoxylin & Eosin (H&E) [24]. We examined the sections under a light microscope (Olympus CX 41, Japan). Although liver and kidney are not a concern for our study aim, they used as a reference to standing if any cytotoxic effect of our ZnO NPs doses.

2.7. Statistical analysis of data

We analyzed the data using IBM SPSS Statistics for Windows, Version 22.0 New York, United States. Data were represented as mean ± standard deviation (SD) and statistically analyzed using one-way Analysis of Variance (ANOVA). To test the intergroup homogeneity, we used Duncan's test. Statistical significance was set at $p < 0.05$. We used Pearson's correlation linear regression to test whether any correlation exists between the expression of steroidogenic enzymes and serum testosterone.

3. Results

3.1 mRNA gene expression of steroidogenesis-related genes using RT- PCR

The expressions of the steroidogenesis related enzymes (StAR, CY11A1 and 3 β –HSD) are illustrated in table 2 and Fig 1. Our findings recorded that there aren't significant differences of gene expressions among negative and positive control groups ($p > 0.05$). The BaP group showed a highly significant decrease in gene expressions reach to -81.7% for StAR, -61% for CYA11A1, and – 81% for 3 β–HSD when compared to the negative control group ($P < 0.001$). Co-administration supplementation of ZnO NPs with B[a]P recorded a significant increase in the expression of steroidogenesis related enzymes when compared to the BaP group. StAR gene expression significantly increased by 225.6 %; 351%; CY11A1 increased by 167.3% and 207 %, and 3 β-HSD by 301%; 340 in In Bap + ZnONPs 10 and Bap + ZnONPs 30 groups respectively ($P < 0.001$). Pearson's linear correlation between testosterone level and the expression of steroidogenic enzymes are highly significant (table 2-C). Although our results recorded an improvement in gene expressions after toxic exposure to BaP, they still under the negative control levels. These findings showed that ZnO NPs supplementation promoted the expression of steroidogenic enzymes, which inhibited by BaP with fairly good outcome, although it could not bring it back into control.

3.2. Antioxidant/ oxidative stress indicators (Malondialdehyde; MDA and reduced glutathione; GSH)

The results of antioxidant biomarkers including MDA and GSH are illustrated in table 3 and figure 2. Positive control I and II showed significant difference when compared with negative control group ($P<0.05$) . The BaP group recorded a significant increase in the serum level of MDA by 35% and a significant decrease in GSH level by -37.6 % when compared with the negative control ($P<0.05$). While Co-administration of Bap with ZnO NPS (Bap+ZnONPs10; Bap+ZnONPs 30) have an anti- Bap effect as it recorded a corresponding a significant decrease in MDA level by -26.5%, -35.5% and increase in of GSH level by 46.3% and 43.3% respectively compared to BaP group and significantly improved when compared to negative control one as it recorded non significant difference ($P>0.05$).These findings showed that ZnO NPs supplementation recorded antioxidant stress either at the level of negative control comparison or counteracting BaP and returning oxidative stress biomarkers to control levels.

3.3. Testosterone concentration and sperm count

The statistical analyzed data for testosterone level and sperm counts are illustrated in table 4 and figure 3. Although testosterone level recorded there is no significant difference between NC and PCI ($p > 0.05$), a significant increase

in its level was recorded in the PCII group. The data of BaP group recorded significant decrease (-39.2 %) than that of the negative control. Co-administrated groups (Bap+ZnO NPs10; Bap+Zn ONPs 30) reported a significant improvement in testosterone level when compared to the Bap group by 40.5%, 48.9% respectively. But its levels in co-administrated groups still recorded significant difference than NC .

Sperm count recorded that, there aren't significant differences of gene expressions among negative and positive control groups ($p > 0.05$). The BaP group revealed a significant decrease in the sperm count than negative control by -50.2 % ($P<0.001$) . Wherease, The Co-administration groups (Bap+ZnoNPs10; Bap+ZnoNPs 30) recorded a significant increase in sperm count compared to the Bap group with an increase of 59.9 % and 83.1 % respectively ($P<0.001$). However, the sperm counts remained significantly under the negative control group . These results indicated that, ZnO NPs supplementation stimulated testosterone synthesis accompanied by increased sperm counts inhibited by BaP with fairly good outcome, although they remained under the control.

3.4. Histopathological findings

Upon microscopic examination, animals in the negative and positive control groups revealed a normal testicular, kidney, and liver architecture and morphology. Benzo[a]Pyrene treated group alone induced various

histopathological alterations in examined tissues. In testicular tissues, B[a] P causing atrophy of the seminiferous tubules associated with severe vacuolar degeneration and desquamation of spermatogonial cells lining seminiferous tubules, lost integrity of cellular membranes, the atrophy of seminiferous tubules, lack of spermatids and spermatozoa, and altered morphology of spermatogonia and spermatocytes, with the presence of multinucleated spermatid giant cells (Symplast) **(Fig. 4. B&C).**While in the kidney it showed vacuolations of glomerular tufts, severe atrophy and mild degeneration of glomerular tuft associated with an increase in the Bowman's space **(Fig. 5. B)**, congestion of renal blood vessels and degeneration of epithelial cells lining renal tubules severe atrophy and degeneration of glomerular tuft and necrobiotic changes of epithelial lining renal tubules. Liver tissues also showed alteration of the cellular architecture pattern of the hepatic parenchyma associated with hepatic parenchyma cellular disorganization of the hepatocytes and vascular dilation and congestion, some sections showed non occluding thrombus formation , some sections showed leucocytic cells infiltration of portal area with activation of kupffer cells (**Fig. 6. B&C**). Improvements in the histopathological pictures were noticed in three examined organs from rats treated with Nano-Zinc Oxide and B[a]P. The aforementioned pathological alterations are depicted in the photomicrographs (Fig 4, 5& 6).

4. Discussion

Available data conclusively proved that Benzo[α] pyrene reduces male fertility. Zinc oxide can be stated as a multifunctional material due to its unique physical and chemical properties. It is known to be crucial for testosterone synthesis and spermatogenesis. While the studies of ZnO NPs effects on male fertility is still rare, either at the in vitro and in vivo levels. Therefore, this study sought to evaluate the ameliorative effect of ZnONPs supplementation on male fertility in Benzo[α] pyrene exposed rat through molecular, biochemical and histological impaction on testis and epididymis .

In our study , Benzo[a] Pyrene induced severe oxidative stress which significantly increased MDA and decrease GSH levels ($P<0.001$) . BaP is one member of PAHs which undergoes to intracellular biotransformation by cytochrome P450 (CYP) enzymes, leading to the production of reactive oxygen species (ROS) comes from reduction of antioxidant enzymes as reduced glutathione. Free radical initiates lipid peroxidation through a chain reaction thus increasing the level of lipoperoxidation product such as MAD [25]. This correlates logically with oxidative stress [12,15] which was consistent with our results .

Regarding ZnO NPs supplementation, it recorded antioxidant stress either at the level of negative control group comparison or counteracting BaP and returning oxidative stress biomarkers to control levels. Zn is a core component of

over 200 metalloenzymes, including antioxidant enzymes and a known protector of sulfhydryl groups; it is also thought to weaken lipid peroxidation, which provides it anti-oxidative stress features [26,27]. ZnO NPs dietary supplementation (10mg /kg /Bw) for nicotine-exposed rats reduces the harmful effects of exposure, through reducing oxidative stress and improvement of male fertility [8]. Beside that, the lower doses of ZnO NPs (10 mg /kg /Bw) have a protective effect on sperm of diabetic rats owing to antioxidant properties as ZnO NPs increase the activity and mRNA expression levels of SOD, CAT, and GSH and decreased MDA levels in testicular tissue[9]. On the contrary, Hussein et al. Recorded that ZnONPs decreased Antioxidant capacity and increased oxidative stress inducing severe reproductive toxicity in male rats . The result come from the doses used by authors as they used 100 & 400 mg/kg /BW which lead to this oxidative stress [28]. Again, we can go back to what has been stated in Pinho's review, The effects of ZnO NPs depend on the size, the concentration used, the morphology, the surface area. These ZnO NPs at low concentrations it act as antioxidant agents, while reactive oxygen species (ROS) can generate and induce apoptosis at high concentrations [5].

Testosterone level in our study was emphasized by the manifested steroidogenesis related enzymes using Pearson's linear correlation between testosterone level and the expression of steroidogenic enzymes, which recorded highly significant correlation . Cholesterol is transferred from outside to inside the mitochondrial membrane depending mainly on the pivotal role of the

Steroidogenic Acute Regulatory (StAR) protein. cholesterol undergoes oxidation by mitochondrial cytochrome P450 oxidase (P450scc; CYP11A1) and converts to pregnenolone. The pregnenolone is oxidized by 3β-HSD and produces androstenedione which is reduced by other enzymes and produces testosterone [29]. In the BaP group, both of Testosterone and the expression of steroidogenic enzymes were significantly than negative control group ($P<0.001$). Our results are in harmony with previous observations recorded that StAR, CY11A1 and 3β HSD can be regulated by endogenous and exogenous agents, including environmental toxins like B[a]P which affects LH stimulated Leydig cell and serum testosterone production [12,30,31]. At the same context, under oxidative stress conditions, ROS activates stress leading to decrease StAR, CY11A1 and 3β-HSD gene expressions [13]. This implies a potent strength opposing correlation between oxidative stress and testicular steroidogenesis [32,33].

Regarding Co-administrated groups showed an increasing the testosterone level in parallel with an improvement in gene expression of steroidogenic enzymes in comparing with BaP group . This agrees with that recorded previously by Le et al. who stated that, ZnO NPs induces up-regulation of the genes and increasing gene expression dependent on the exposure time and concentration [34]. Recently, Bara and his co-authors [35] examined the direct effect of ZnO NPs in vitro using different concentrations on mouse testicular Leydig cells (TM3) and recorded significant amplification of the expression of steroidogenic enzymes

342 (STAR and CYP11A1). Recently, Mohamed et al. have reported that ZnO NPs
343 supplementation with a dosage of 10mg/kg/BW caused an increase in testicular
344 gene expression of StAR and cytochrome P450scc as in parallel with the level of
345 testosterone in nicotine exposed rats [8]. In contrast Tang et al. [36] reported that ZnO
346 NPs decrease testosterone production through the downregulation of StAR. This
347 difference come from the dose which the author used (50, 150, and 450 mg/kg).
348 Although several literature discusses the protective effect of ZnO NPS against
349 drugs or toxic substances, to date, the molecular mechanism of ZnO NPs is still
350 absence, especially in vivo.

351 Sperm count showed an significant decrease in B[a]P group ($P<0.001$) as
352 compared to negative control one. This result is predictable as testosterone levels
353 highly decreased. Testosterone is the androgen in the testis that
354 supports spermatogenesis [37]. BaP as an oxidative stress inducer, it damages DNA
355 in the sperm nucleus and increases apoptosis at a specific stage of the germinal
356 cycle [38–40]. Our results were further supported by histopathological changes in
357 testis as showed the deleterious effects of B[a]P on the testis. The alteration in
358 seminiferous tubules architecture, altered morphology of spermatogonia and
359 spermatocytes, and atrophy of seminiferous tubules showed B[a]P interferes with
360 the process of spermatogenesis [41,42].

361 ZnO NPs Co-administration improves sperm count and histopathological
362 findings. These results agree with other investigations recorded that the

363 administration of ZnO NPs prevented testicular toxicity and sperm damage via
364 an antioxidant mechanism, against doxorubicin [7,43] and Nicotine in adult rats [8].

365 Our histopathological findings, which including nephrotoxic adverse effects
366 of B[a]P, including degeneration, atrophy of glomerular tuft and necrotic lining
367 epithelium and disorganization of the hepatic parenchyma, necrosis, and
368 leucocytic cells infiltration agree with previously published studies [44–46]. And can
369 be interpreted by a previously published study who recorded that increasing free
370 radical and ROS production by BaP reinforce tissue damages and considered the
371 central causative factor responsible for pathological finding through membrane
372 lipid peroxidation and DNA mutations [47].

373 Nano-Zinc Oxide is known for its antioxidant and anti-inflammatory
374 properties. This antioxidant activity mainly occurs through neutralizing and
375 scavenging free radicals [48–51]. This concept is supported by its ability to protect
376 cell membrane integrity by increasing the antioxidant enzyme levels and
377 decreasing MDA and free radical levels [52]. Zinc has anti-apoptotic properties that
378 guard cells against different pro-apoptotic molecules [53]. Our histopathological
379 findings are in line with these concepts as all examined tissues recorded normal
380 histology like the control group. This is a sign that ZnO NPs are helpful for tissue
381 regeneration to reverse damage caused by Benzo [a]Pyrene.

382

Conclusion

Our findings at the designed doses and with the properties of nanoparticles in this study concluded that ZnO NPs have an obvious ameliorative effect against B[a] P through decreasing oxidative stress and increasing expression of steroidogenic enzymes, repair tissue abnormalities which may progress a new hope in both reproductive toxicology and nanomedicine fields. Further researches are needed to discover its different mechanisms in improving male fertility.

Table (1): Primers sequences for RT-PCR

Gene	**Oligonucleotides sequence (5′–3′)**	**Accession Number**
StAR	F: TCT CTA GTG TCT CCC ACT GCA TAG C R: TTA GCA TCC CCT GTT CG TAG CT	NM_011485.5
CYP11A1	F: ACAT GGC CAA GAT GGT ACA GTT G R: ACG AAG CAC CAG GTC ATT CAC	NM_019779
3β-HSD	F: ACAT GGC TCT GGG AGT TAT AAG GT R: TTA GTG ACT GGC AAG GCT TCT G	NM_008293
B-actin	AGA AGA TCT GGC ACC ACA CC TAC GAC CAG AGG CAT ACA GG	NM_007393.5

† **Abbreviations: F**: forward primer; **R**: reverse primer. **StAR**; steroidogenic acute regulatory protein; **CYP11A1**- P450scc-cholesterol side-chain cleavage enzyme ; **3 β -HSD** -3β-hydroxysteroid dehydrogenase-1.

Tables (2) Effect of Zinc Oxide Nanoparticles on the relative expression of Steroidogenic enzymes in Bap-challenged male rats.

genes / Groups	StAR	CY11A1	3β-HSD
Negative Control (NC)	1.006 ± .0115	1.010 ± .0173	1.000 ± .000
ZnO NPs 10 (PC)	1.016± .015	1.006± .011	1.010± .010
ZnO NPs 30 (PC)	1.020 ± .010	1.003± .005	1.010 ± .010
Bap	0.183± .045 *	0.621 ± .052 *	0.190 ± .113 *
Bap + ZnO NPs 10	0.596 ± .070 *	1.660 ± .150 *	0.763 ± .028 *
Bap+ ZnO NPs 30	0.826 ± .109 *	1.910 ± .120*	0.836.075 *

A- Statistical comparison among groups using ANOVA test

† Values are represented as mean ± standard deviation (SD).

‡ Values with superscript * within the same column means a significant difference from NC group at $P < 0.05$.

B. Statistical comparison among B[a]P and co-administrated groups using ANOVA test

genes / Groups	StAR	CY11A1	3β-HSD
Bap	0.183± .045 *	0.621 ± .052 *	0.190 ± .113 *
Bap + ZnO NPs 10	0.596 ± .070 *	1.660 ± .150 *	0.763 ± .028 *
Bap+ ZnO NPs 30	0.826 ± .109 *	1.910 ± .120*	0.836 ± .075 *

† Values are represented as mean ± standard deviation (SD).

‡ Values with superscript * within the same column means a significant difference from Bap group at $P < 0.05$.

§ **Abbreviations**: StAR ; Steroidogenic Acute Regulatory protein ; CYP11A1; P450Scc cholesterol side-chain cleavage enzyme ; 3 β -HSD - 3β-Hydroxysteroid Dehydrogenase 1; Bap- Benzo [a] Pyrene ; ZnO NPs- zinc Oxide Nanoparticles.

C- Pearson's correlation analysis between the expression of Steroidogenic enzymes and Testosterone level .

	StAR	CY11A1	3β-HSD
Pearson correlation coefficient with testosterone	r = 0.975	r = 0.392	r = 0.985
P value	.000	.022	.000

¶ StAR ; steroidogenic acute regulatory protein ; CYP11A1- cholesterol side-chain cleavage enzyme ; 3 β -HSD - 3β-hydroxysteroid dehydrogenase. P significant at < 0.05.

Table (3) Effect of ZnO NPs on oxidative stress biomarkers in serum of B[a]p-challenged male rats.

A. Statistical comparison among groups using ANOVA test

Parameters / Groups	MDA nmol /ml	GSH mg/dl
Negative Control (NC)	7.52 ± 0.05	2.18 ± 0.10
ZnO NPs 10 (PC I)	6.10 ± 0.30 *	2.77 ± 0.12*
ZnO NPs 30 (PC II)	6.24 ± 0.14*	2.80 ± 0.14 *
Bap	10.17 ± 0.21 *	1.36 ± 0.17 *
Bap + ZnO NPs 10	7.47 ± 0.02	1.99 ± 0.03
Bap + ZnO NPs 30	6.55 ± 0.03 *	1.95 ± 0.02

† Values are represented as mean ± standard deviation (SD).

‡ Values with superscript * within the same column means a significant difference from NC group at $P < 0.05$.

B- Statistical comparison between B[a]P and supplemented groups using ANOVA test

Parameters / Groups	MDA nmol /ml	GSH mg/dl
Bap	10.17 ± 0.21	1.36 ± 0.17
Bap + ZnO NPs 10	7.47 ± 0.02 *	1.99 ± 0.03*
Bap + ZnO NPs 30	6.55 ± 0.03 *	1.95 ± 0.02*

† Values are represented as mean ± standard deviation (SD).

‡ Values with superscript * within the same column means a significant difference from Bap group at $P < 0.05$.

§ **Abbreviations:** MDA, Malondialdehyde ; GSH; reduced glutathione, Bap- Benzo [a] Pyrene ; ZnONPs- zinc Oxide Nanoparticles.

Table (4): Effect of zinc Oxide nanoparticles on serum testosterone and sperm counts in Bap-challenged male rats.

A. Statistical comparison among groups using ANOVA test

Groups	Sperm count ($X10^6$/ml)	Testosterone ng /dl
Negative Control (NC)	73.66 ± 6.51	5.32± 0.18
ZnO NPs 10 (PCI)	68.83 ± 3.76	5.49 ± 0.12
ZnO NPs 30 (PCII)	73.83 ± 6.30	5.88 ± 0.11 *
Bap	36.67 ± 2.65 *	3.23 ± 0.05 *
Bap + ZnO NPs 10	58.67 ± 7.44 *	4.54 ± 0.04 *
Bap+ ZnO NPs 30	67.17 ± 3.25*	4.81± 0.14 *

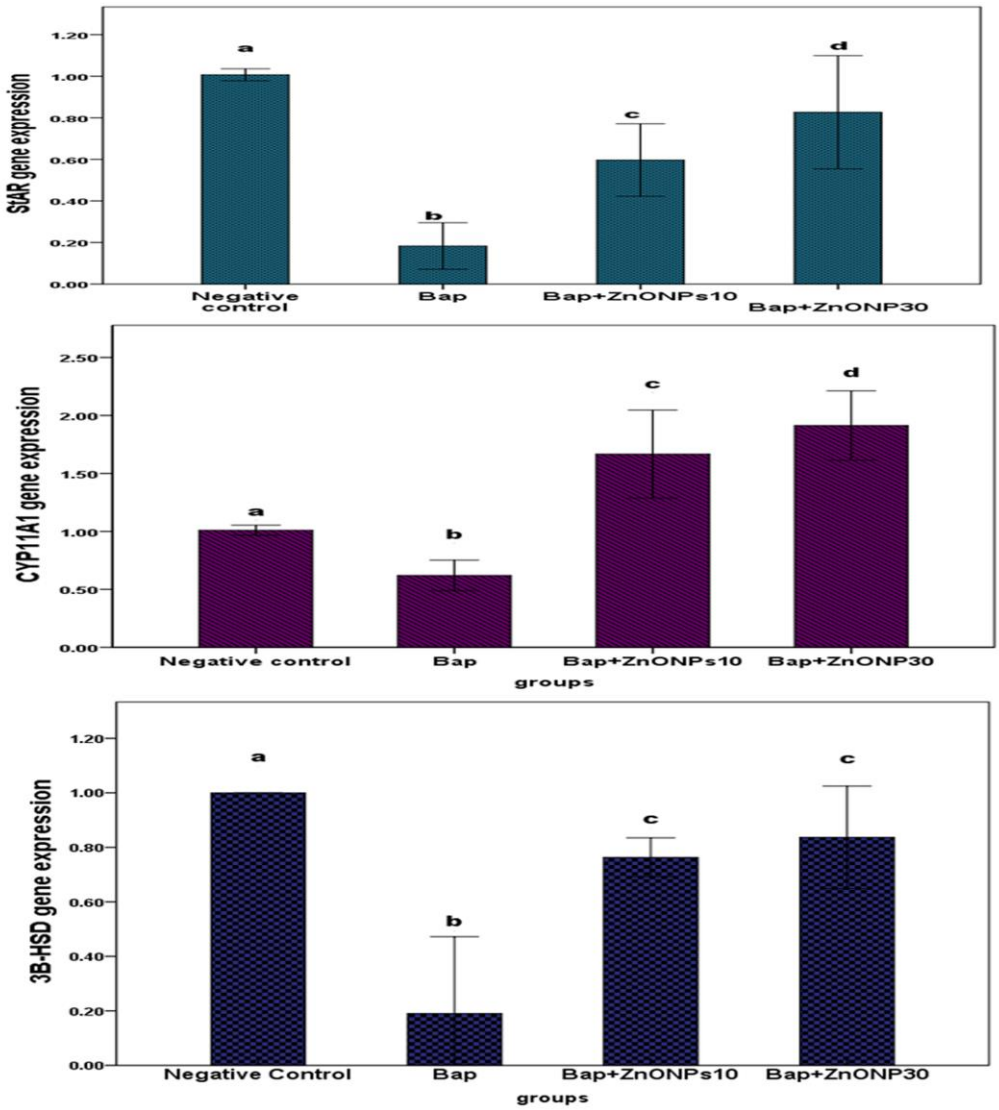
† Values are represented as mean ± standard deviation (SD).
‡ Values with superscript * within the same column means a significant difference from NC group at $P < 0.05$.

B. Statistical comparison among B[a]P and Co-administrated groups using ANOVA test

Groups	Sperm count ($X10^6$/ml)	Testosterone ng /dl
Bap	36.67 ± 2.65 *	3.23 ± 0.05 *
Bap + ZnO NPs 10	58.67 ± 7.44 *	4.54 ± 0.04 *
Bap+ ZnO NPs 30	67.17 ± 3.25 *	4.81± 0.14 *

† Values are represented as mean ± standard deviation (SD).
‡ Values with superscript * within the same column means a significant difference from Bap group at $P < 0.05$.

§ **Abbreviations** : Bap- Benzo [α] Pyrene ; ZnO NPs- zinc Oxide Nanoparticles.



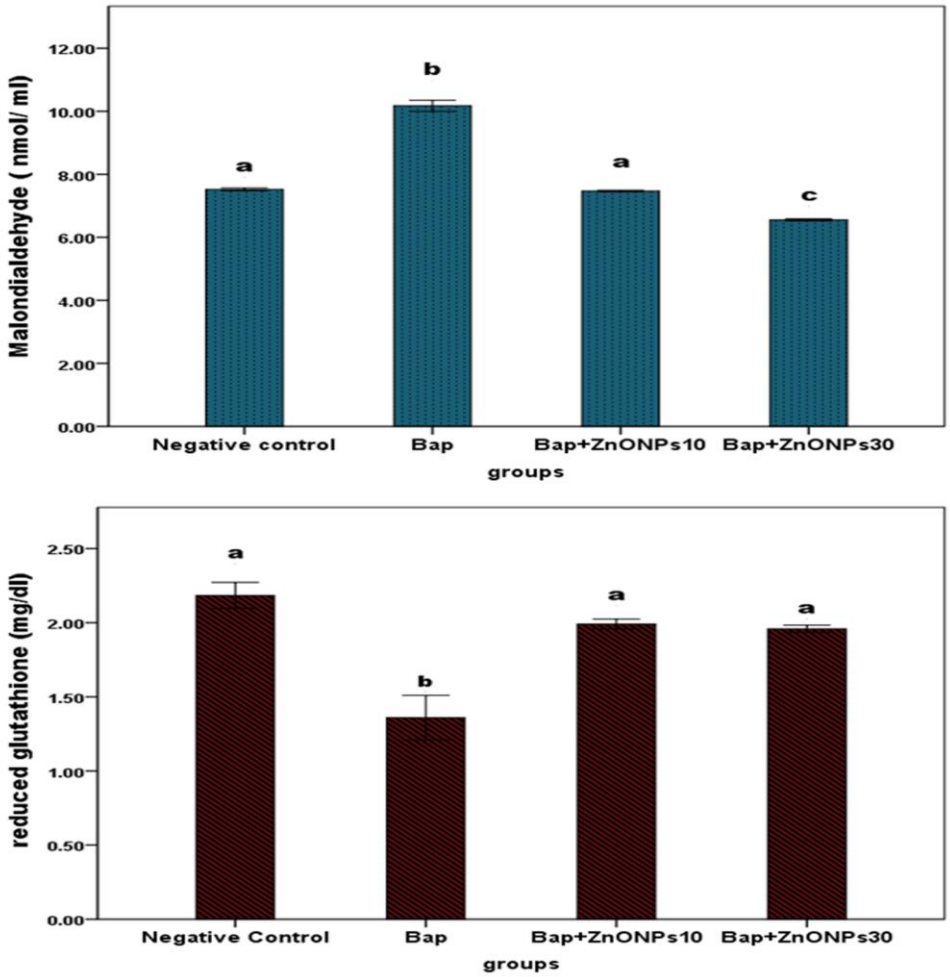
487

488 **FIGURE 1: The Effect of Zinc Oxide Nanoparticles against Benzo [a] pyrene challenged rats.**
489 Results are represented as expression controlled by B actin. Bap group showed highly significant
490 decrease the expression of all steroidogenic enzymes when compared to the negative control group.
491 Co-administration of ZnO NPs with B[a] P resulted in a significant increase in the expression of them
492 when compared with the Bap group although it could not bring it back into control.

493 # Means with different superscripts (a, b, c and d) among different groups are significant at $P < 0.05$.

494

495 **Abbreviations:** StAR; Steroidogenic Acute Regulatory protein; CYP11A1; P450 Scc - cholesterol
496 side-chain cleavage enzyme; 3 β -HSD - 3β-Hydroxysteroid Dehydrogenase 1; Bap- Benzo [a]
497 Pyrene; ZnO NPs- Zinc Oxide Nanoparticles.



508

509 **FIGURE 2: The antioxidant Effect of Zinc Oxide Nanoparticles (ZnO NPs) against Benzo**
510 **[a] pyrene (Bap) challenged rats.** The Bap group significantly increased the serum level of
511 Malondialdehyde (MDA) accompanied by significant decrease level of reduced glutathione (GSH) when
512 compared with negative control. While Co-administration of Bap with ZnO NPS recorded
513 correspondingly significant decrease in MDA and increase in serum level of GSH which counteract
514 the effect of Bap and back their levels to normal levels especially in GSH.

515 Means with different superscripts (a, b and c) in different groups are significant at $P < 0.05$.

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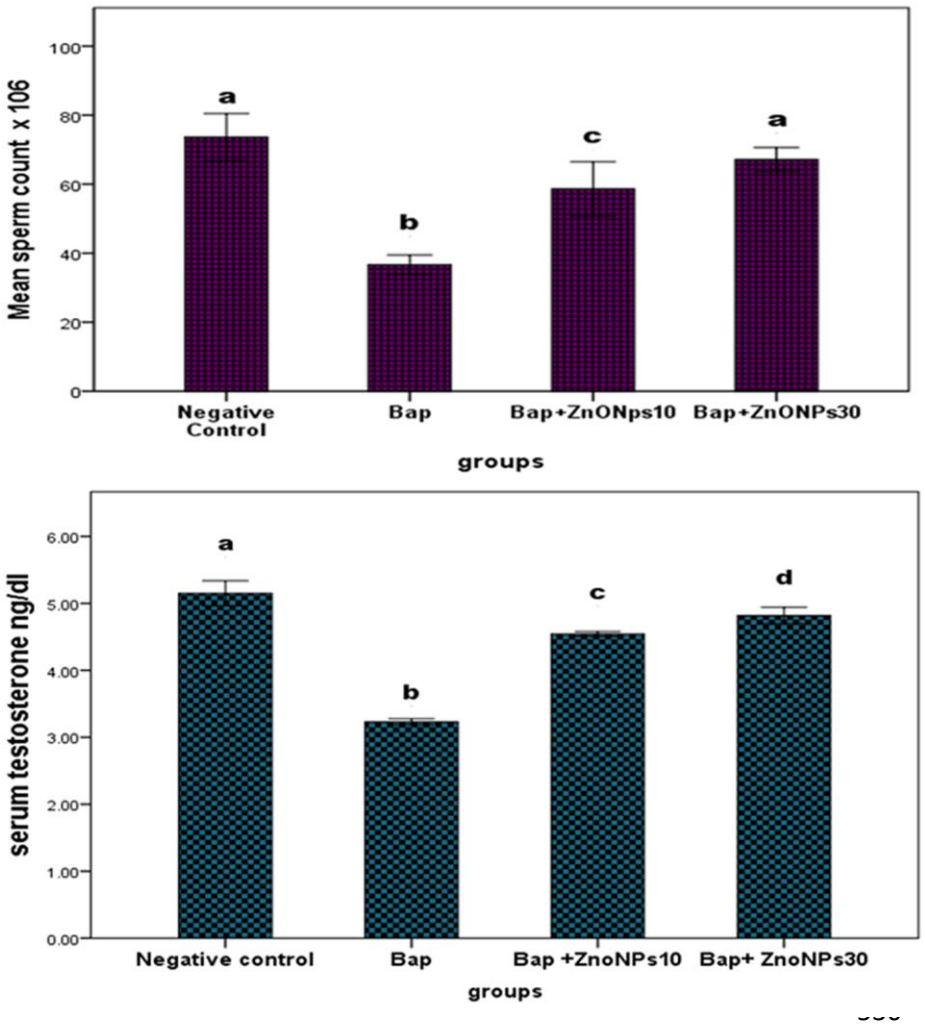
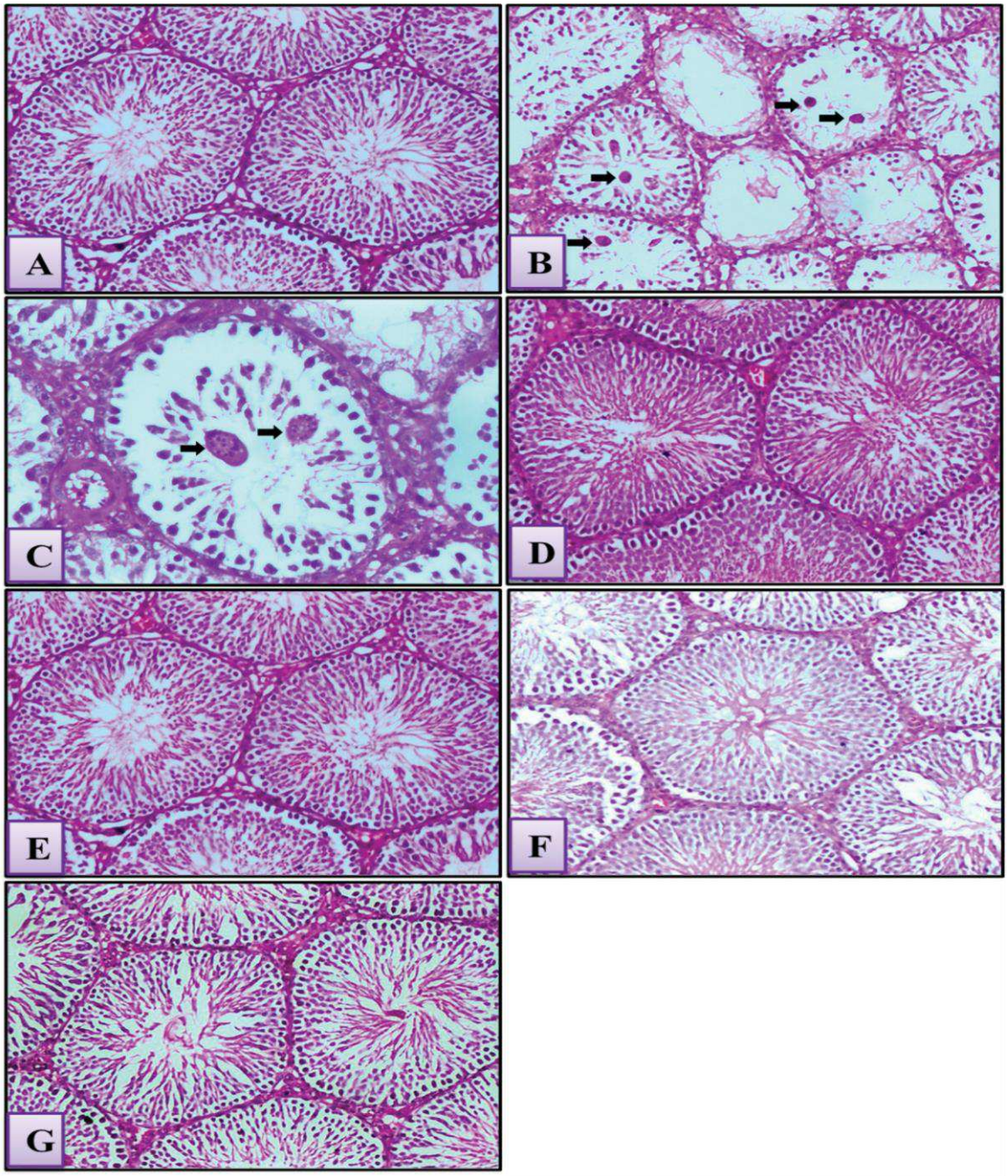


FIGURE 3: Effect of zinc Oxide nanoparticles (ZnO NPs) on serum testosterone and sperm count in Benzo[a] pyrene (Bap)-challenged male rats. Testosterone and sperm count are significantly decreased in B[a]P group compared with the negative control. Co-administrated groups recorded a significant increase in testosterone concentration by nearly one third and sperm count by nearly half and two forth respectively when compared with the Bap group proving that ZnO NPs can guard sperm against toxic effect of B[a]P .

Means with different superscripts (a, b ,c and d) between different groups are significant at $P < 0.05$.



542

543 **FIGURE 4. Photomicrograph** of testis of control rat showing no histopathological change (A). In
544 B[a]P treated group shows severe desquamation of spermatogonial cells in testis of male rat **(B)**. and
545 degeneration and pyknosis of spermatogonial cells associated with giant cells formation (Symplast)
546 (Arrows) **(C)** (H & E X 200). **D and E** Co-administration of Nano-Zinc Oxide and B[a]P improved these
547 histopathological alterations. **F and G** show normal testicular section in male rats treated with Nano-
548 Zinc Oxide (H & E X100).

549

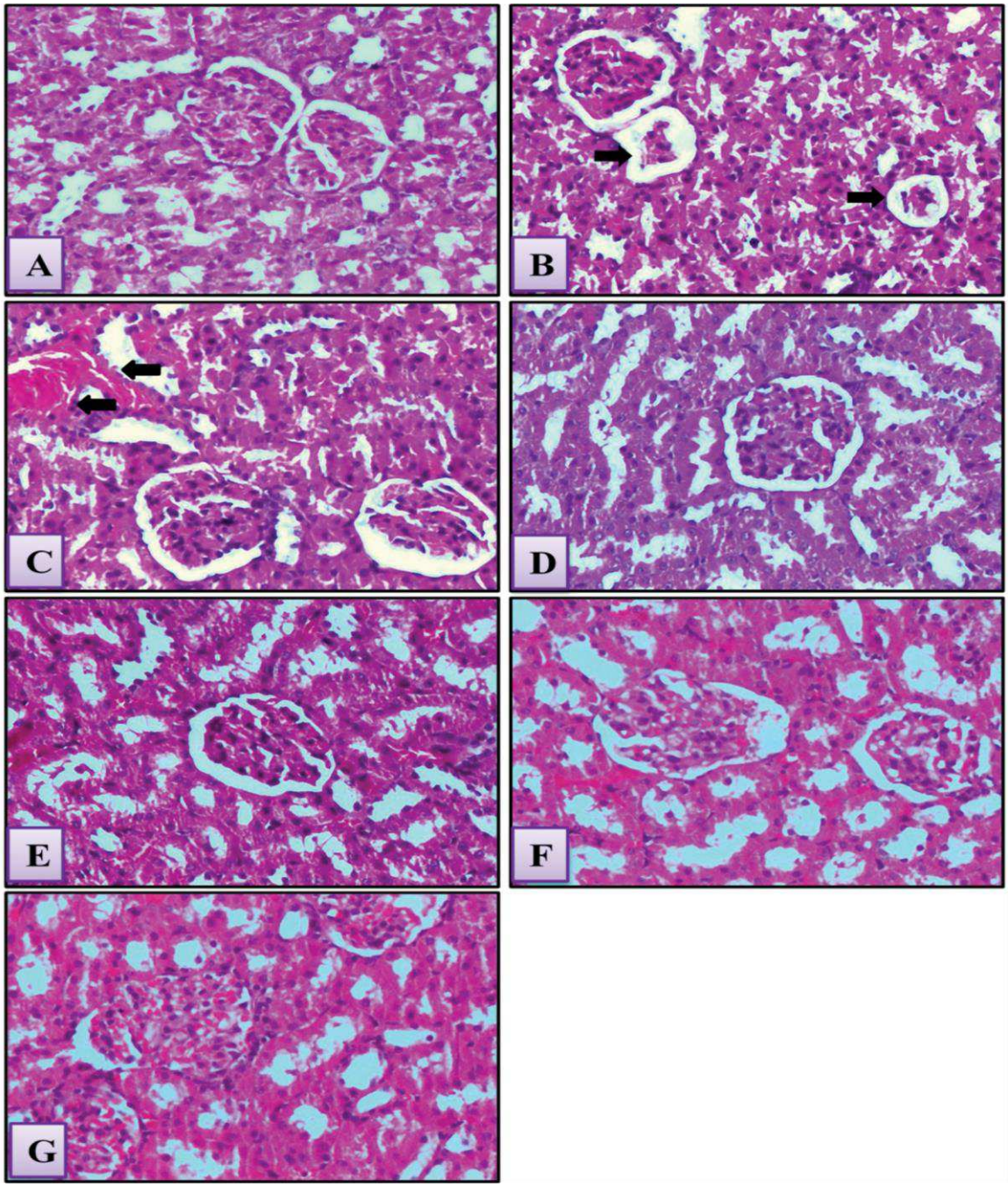


FIGURE 5 . Photomicrograph of Kidney in negative control rat showing no histopathological change **(A).** Benzo [a] pyrene group shows severe atrophy and degeneration of glomerular tuft (Arrow) with vacuolization and necrobiotic changes of epithelial lining renal tubules **(B)** and **(C)** shows necrobiotic changes of epithelial lining renal tubules associated with severe congestion (Arrows) . **D** and **E** show normal histologic features in groups exposed to nano-Zinc Oxide and B[a]P (H & E X200). **F and G** show normal histology in male rats treated with nano-Zinc Oxide. (H & E X200).

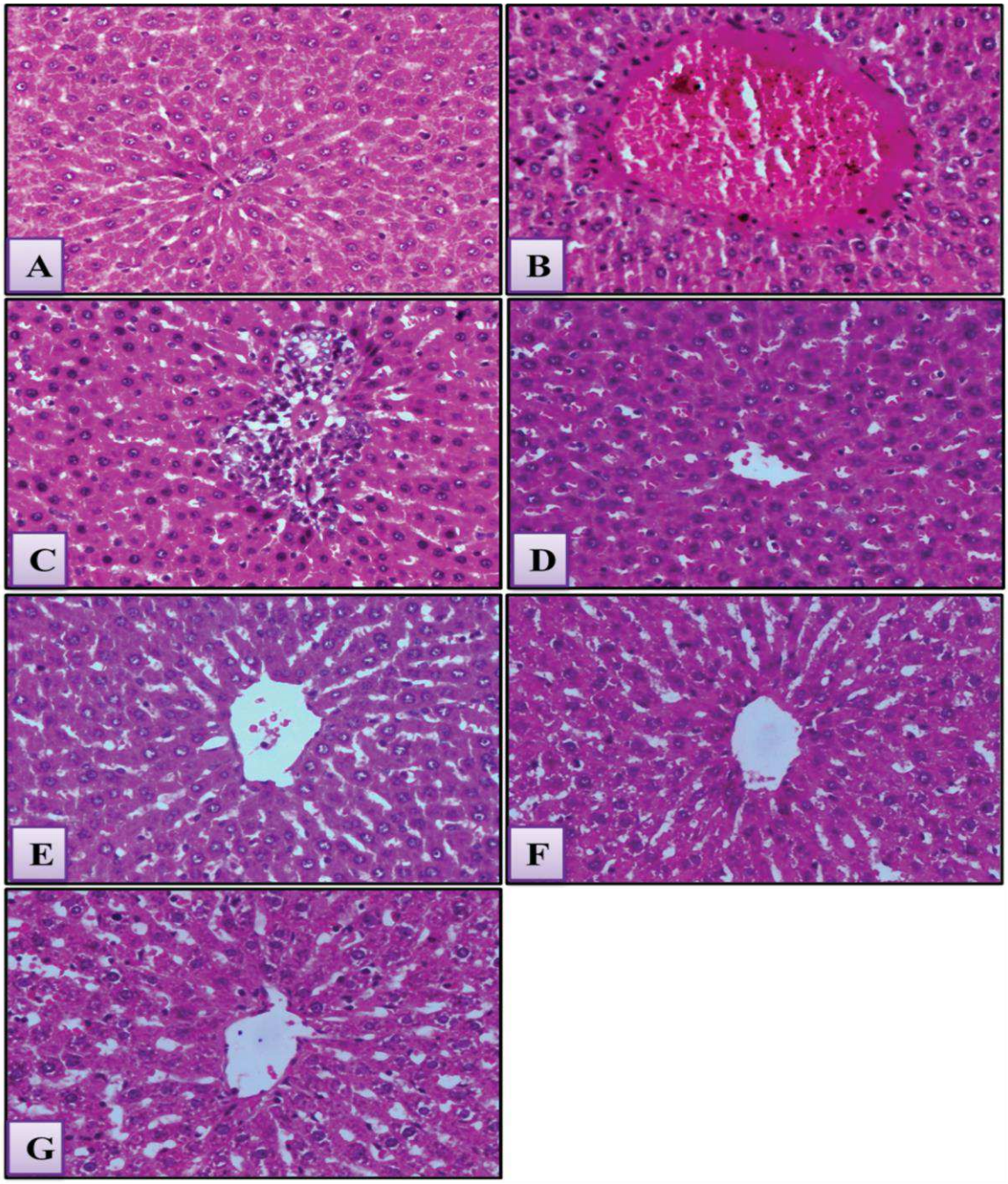


FIGURE 6 . Photomicrograph of Liver of negative control rat showing no histopathological change **(A)**. In Benzo (a) pyrene **(B)** shows disorganization of the hepatocytes and vascular dilation, congestion and non-occluding thrombus associated with thickening of blood vessel wall (H & E X100). And also shows degenerative changes of hepatocytes associated with leucocytic cells infiltration **(C)**. **D and E** show normal hepatic tissue in male rat exposed to nano-Zinc Oxide and B[a] P. **F and G** show normal hepatic histology in male rats treated with nano-Zinc Oxide (positive control groups) (H & E X200).

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Additional Information

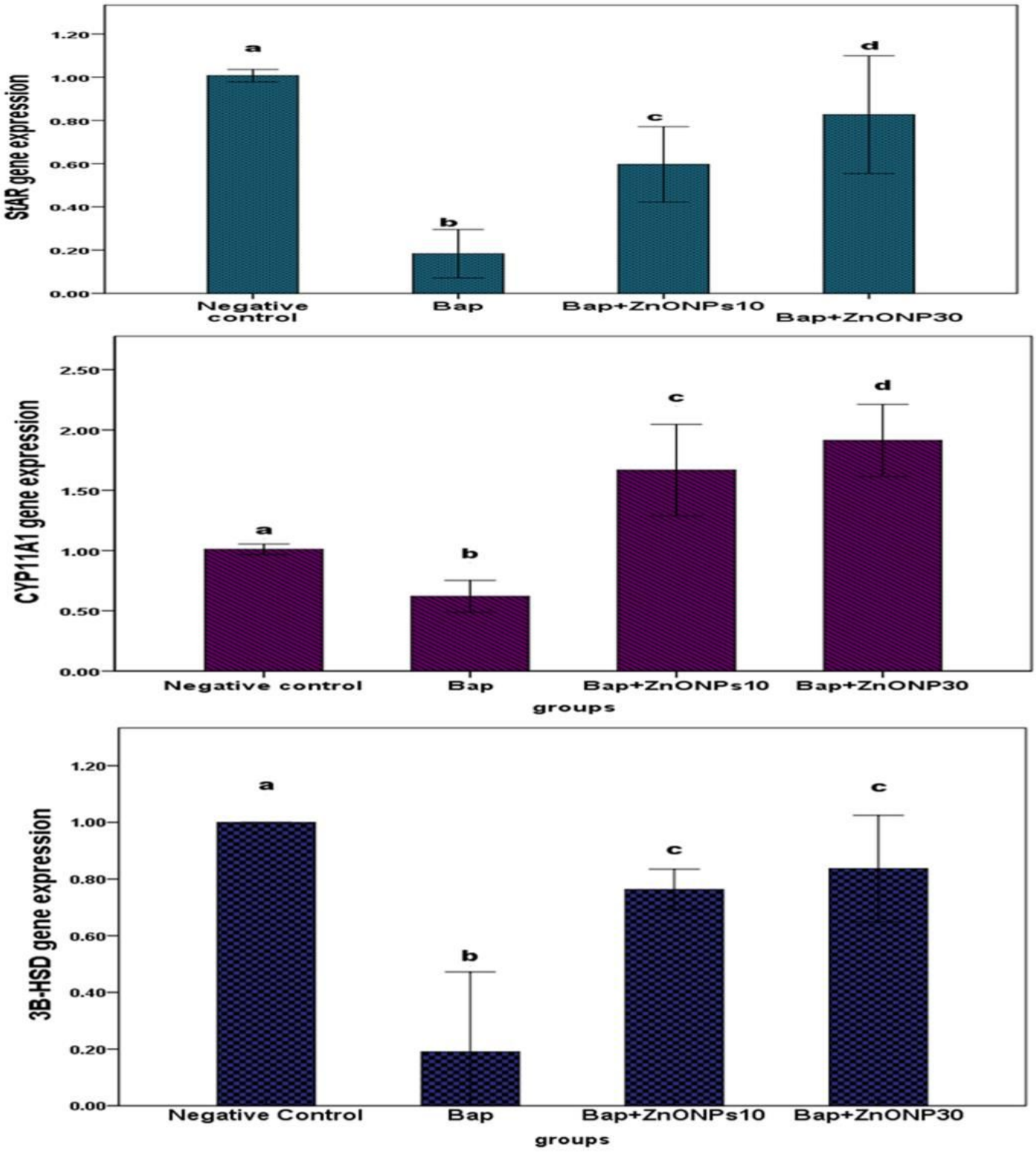
Author contributions statements

Niveen M Daoud : designed and wrote edited the final manuscript text, analyzed the data statistically, prepared figures and tables. **Mohamed S Aly:** Prepared and examined tissues for histopathology; determined sperm counts**;** wrote this part in the manuscript. **Naglaa A Ali**: treated and observed The experiment animals, determined hormonal and biochemical parameters and wrote draft preparation. **Omaima H Ezzo; Niveen M Daoud and Naglaa A Ali**: shared in the experiment's funding and gene expression detection. All Authors reviewed the manuscript.

competing interests statement

We declare that the authors have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Figures



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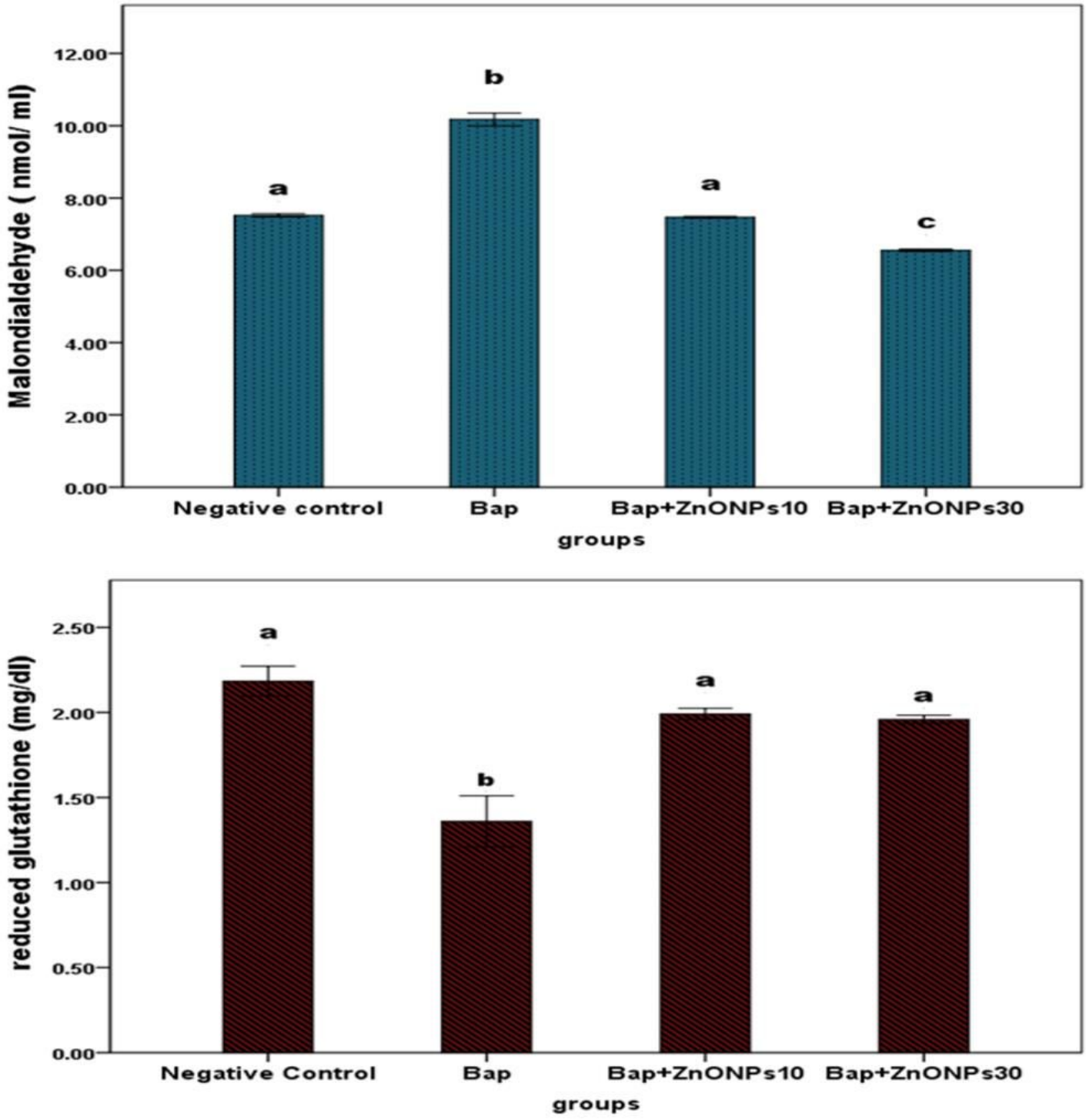

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The antioxidant Effect of Zinc Oxide Nanoparticles (ZnO NPs) against Benzo [a] pyrene (Bap) challenged rats. The Bap group significantly increased the serum level of Malondialdehyde (MDA) accompanied by

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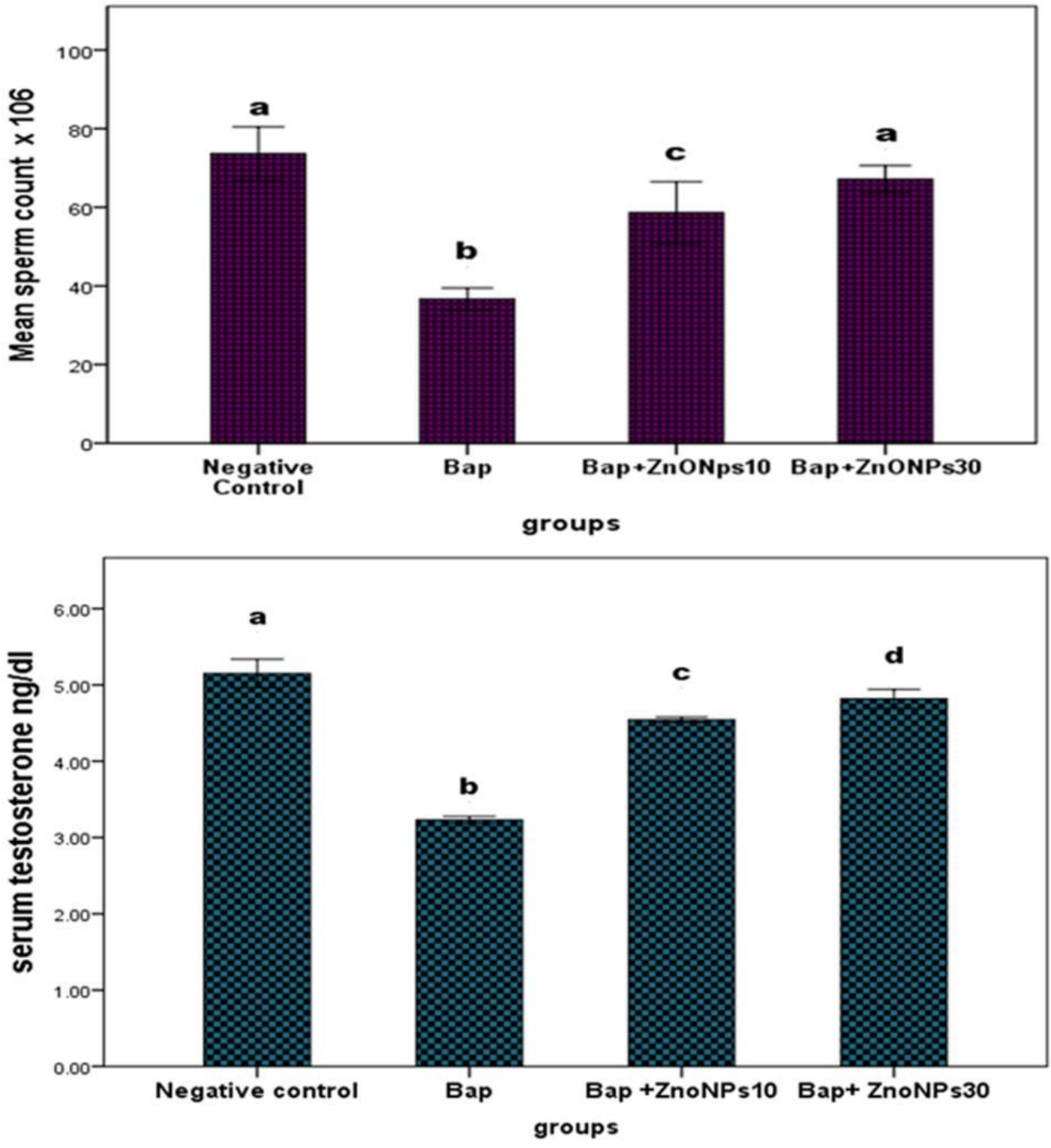

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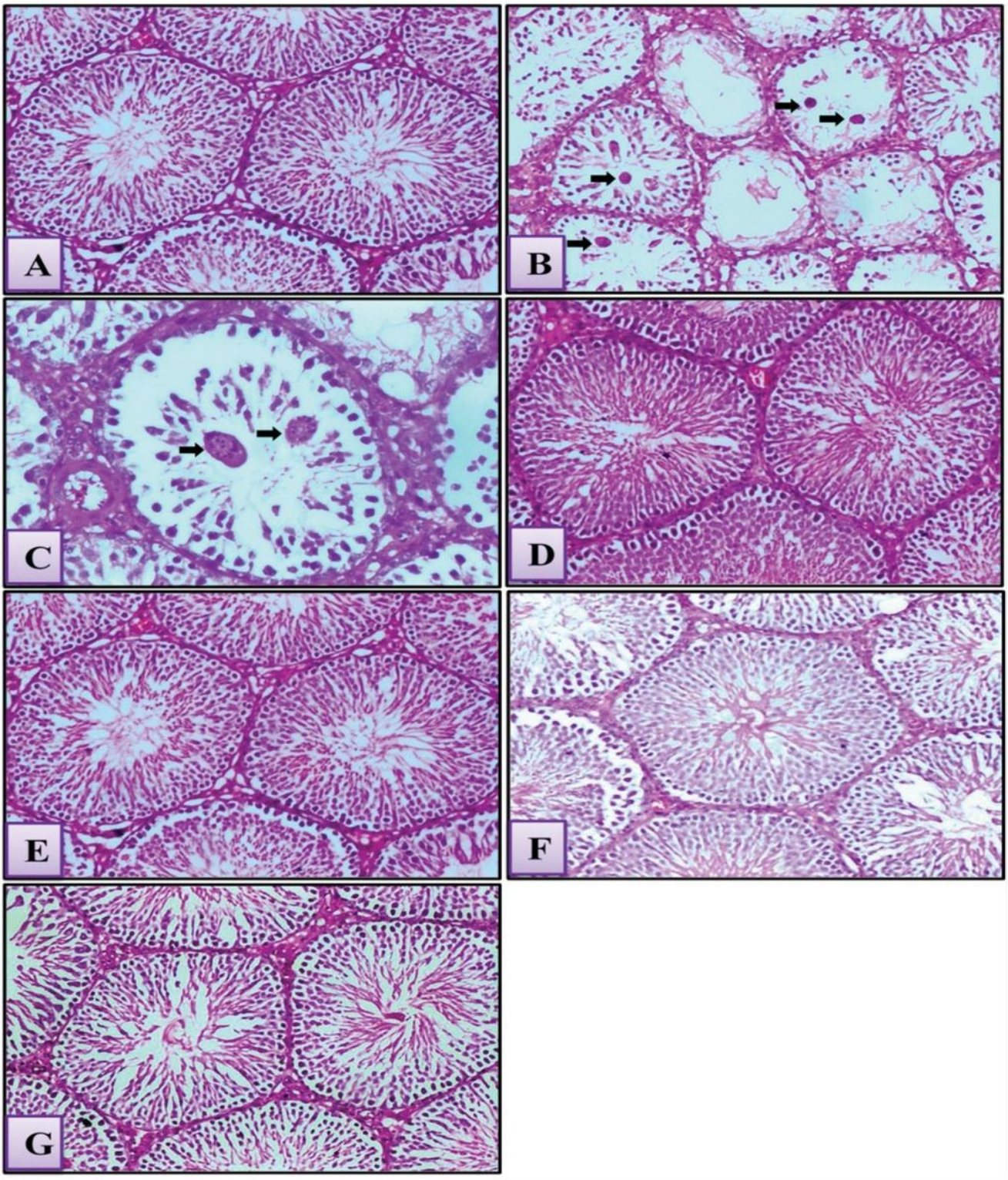

Figure 4

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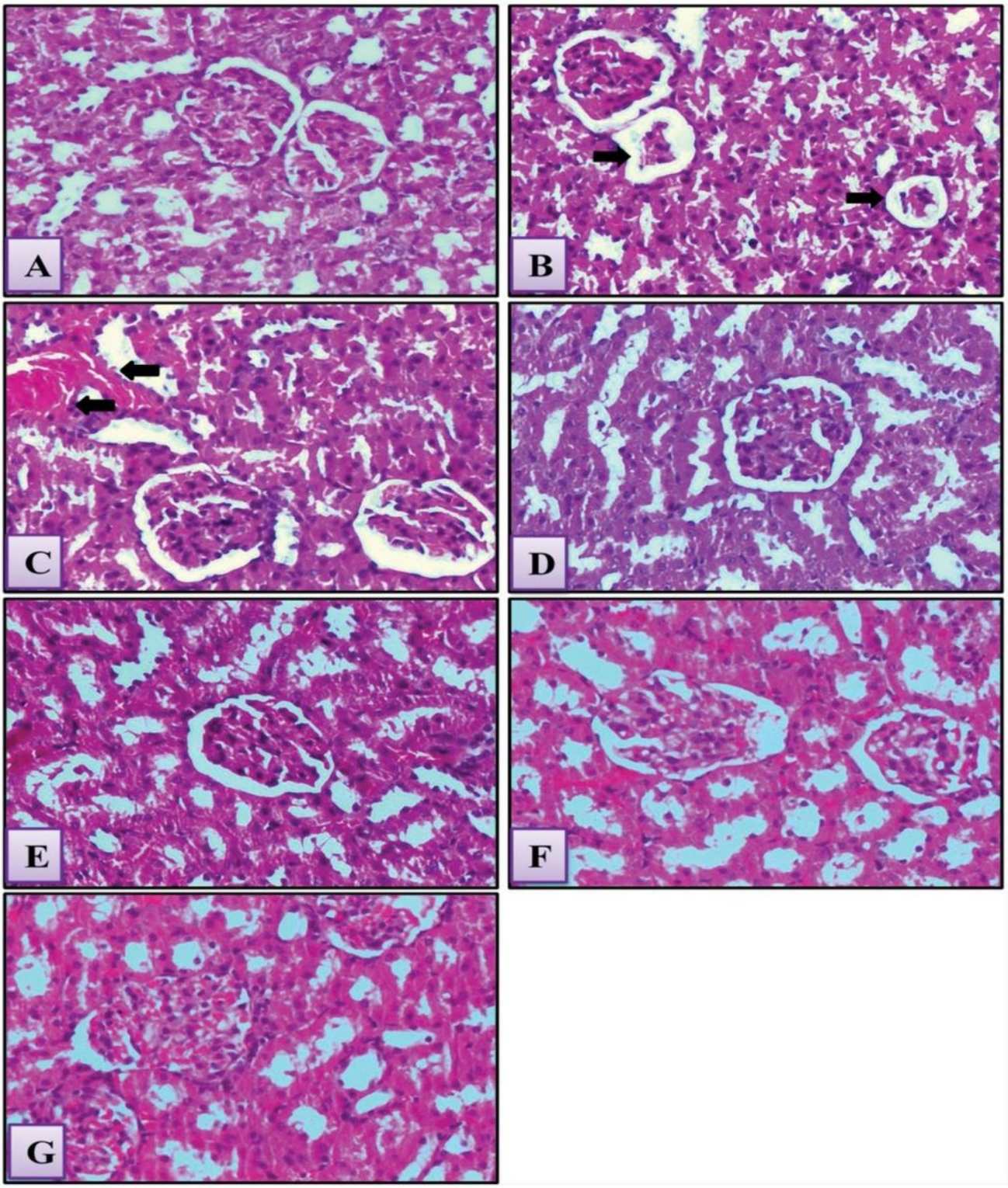

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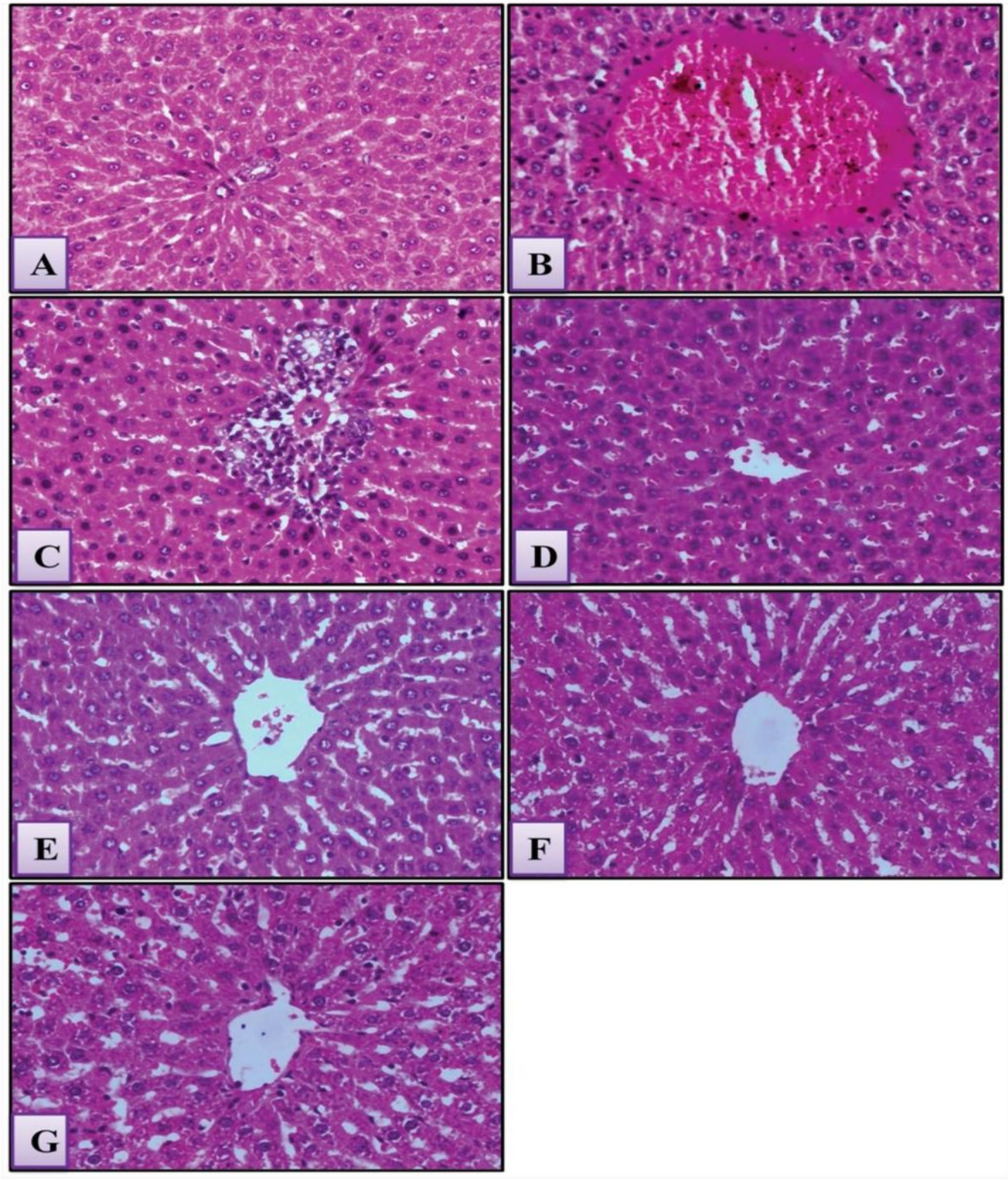

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Photomicrograph of Liver of negative control rat showing no histopathological change (A). In Benzo (a) pyrene (B) shows disorganization of the hepatocytes and vascular dilation, congestion and non-occluding thrombus associated with thickening of blood vessel wall (H & E X100). And also shows degenerative changes of hepatocytes associated with leucocytic cells infiltration (C). D and E show normal hepatic tissue in male rat exposed to nano-Zinc Oxide and B[a] P. F and G show normal hepatic histology in male rats treated with nano-Zinc Oxide (positive control groups) (H & E X200).