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RESEARCH ARTICLE

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Cefepime and diclofenac sodium combined treatment-potentiated multiple organ injury: Role of oxidative damage and disrupted lipid metabolism

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Abstract

Concurrent exposure to antimicrobial and nonsteroidal anti-inflammatory drugs (NSAIDs) is usually inevitable in most infections and postsurgery. Consequently, the present study was designed to assess the intertwining impact of coadministration of cefepime (CP, a wide spectrum antibiotic) and diclofenac sodium (DF, an NSAID) on rat's liver, kidney, and testes. Rats received saline, CP (180 mg/kg/day, IM), DF (10 mg/kg/day, IM), or a combination of CP and DF. After 14 days, CP or DF induced tissue damage expressed by marked biochemical alterations in hepatic and renal function tests. Besides this, disrupted lipid metabolism and testosterone levels along with significant histological changes in hepatic, renal, and testicular tissues were noticed. A significant increase in malondialdehyde and decreases in superoxide dismutase and catalase activities alongside significant upregulated caspase 3

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; COVID-19, Coronavirus Disease 2019; COX, cyclooxygenase; CP, cefepime; DAB, 3,3-diaminobenzidine tetrahydrochloride; DF, diclofenac sodium; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; LPO, lipid peroxidation; MDA, malondialdehyde; NSAID, nonsteroidal anti-inflammatory drug; PBS, phosphate buffer saline; PCoA, principal coordinate analysis; ROS, reactive oxygen species; SOD, superoxide dismutase.

expression in tissues following CP or DF treatment suggested a bearable influence of oxidative stress, lipid peroxidation, and cell death. Accordingly, the simultaneous therapy of CP and DF evoked more obvious tissue damage than their individual treatment. Overall, data concluded that concurrent use of CP and DF in medical practice is a worrisome matter, so it should be done cautiously to avoid synergistic deleterious outcomes.

KEYWORDS

caspase 3, combined toxicity, hepato-renal toxicity, oxidative stress, testicular damage, testosterone

1 | INTRODUCTION

Antibiotics are at times fortuitously prescribed for patients who are already taking anti-inflammatory medications.[1] In contrast, the simultaneous prescription of both antibiotics and anti-inflammatory medications is commonly followed in most cases as an excellent therapeutic approach.[2] As bacterial infections are usually accompanied by pain and inflammatory reactions, this combination can together overwhelm the pathogens and interrupting the sequences of inflammation.[3] Practically, the synchronous exposure of both categories is inevitable. Cefepime (CP), together with diclofenac sodium (DF) is an example of both remedies' concurrent use.

CP is an extended-spectrum 4th generation antimicrobial cephalosporin that has a powerful action against Gram-positive and -negative microbes.[4] In spite of growing resistance to antibiotics medications, CP is still a potent agent used to fight severe bacterial infections resistant to other drugs. It is clinically applied in a various array of infections such as pneumonia as well as intra-abdominal, skin, urinary tract, and hospital-acquired infections.[5,6] Increasing evidence in preceding studies reported that CP therapy can cause renal insufficiency,[4] reproductive toxicity,[7] neurological disorder, and hepatic toxicity.[6,8] A growing body of literature suggested that CP-induced toxicities probably caused a result of enormous generation of reactive oxygen species (ROS) and oxidative stress that encompass severe damages to several host molecules, including lipids, proteins, and DNA, subsequently mitochondrial perturbations, and eventually apoptosis.[5,9]

DF is a nonsteroidal anti-inflammatory drug (NSAID) which extensively used to alleviate fever, pain, immune-mediated inflammatory reactions, and febrile conditions that are usually accompanying bacterial and viral infections. In addition, it is mostly prescribed in the management of autoimmune diseases (e.g., rheumatoid arthritis), osteoarthritis, acute sciatica, degenerative joint disease, dysmenorrhea, and post-surgical operations.[10] DF exerts its therapeutic action by suppressing cyclooxygenase enzymes (COX-1 and COX-2), thence subsequent inhibition of prostaglandins biosynthesis from arachidonic acid.[11] Despite its tremendous effect in medical practice, several adverse effects hamper its use, such as gastric ulceration,[12] hepatotoxicity,[13] nephrotoxicity,[14,15] and testicular damage.[10] It is known that DF is biotransformed in the liver via cytochrome-P450 into highly reactive toxic intermediates, acyl glucuronide, and benzoquinone imine.[16] When these toxic intermediates are formed in excrescence higher than the capacity of the detoxification system, they disrupt the cellular hemostasis by covalently fasten cellular macromolecules and encourage ROS formation; thereby, oxidative damage and lipid peroxidation are initiated.[17,18]

As mentioned above, in many clinical settings, antibiotics and NSAIDs are commonly prescribed concomitantly. Currently, the horrific Coronavirus Disease 2019 (COVID-19) pandemic would be an example where antibiotics and NSAIDs are used to combat secondary bacterial infection and fever, respectively. This dictates more attention to explore the intertwining relationship between antibiotics and NSAIDs.

To the best of our knowledge, a literature survey divulges that, until date, there is no study has specifically focused on the potential impact of combined treatment of DF with the family cephalosporin antibiotics. Moreover, information about CP-induced oxidative stress, apoptosis, and disrupted lipid metabolism is scarce. Therefore, the current study was setup to evaluate the deleterious effects of DF and/or CP administration on hepatic, renal, and testicular tissues in a rat model.

2 | MATERIALS AND METHODS

2.1 | Drugs

Diclofenac sodium, DF (Voltaren, 75 mg/3 ml ampoule) was obtained from Novartis. Cefepime, CP (Maxipime, 1 g vial) was purchased from Bristol Myers Squib.

2.2 | Experimental animal and design

Twenty-eight male Wister albino rats weighing 170–190 g were obtained from the Laboratory Animal Center, Faculty of Veterinary

Medicine, Benha University, Egypt. Rats were harbored for 2 weeks before the commencement of the experiment. All rats were kept under controlled environmental conditions (20–25°C temperature and 45%–55% relative humidity) with free access to a standard commercial diet and water.

After the acclimation, experimental rats were haphazardly allocated into four even groups, seven rats each; Group 1 (Control): rats served as vehicle control and received saline, IM injection. Group 2 (CP): rats received CP at a dose of 180 mg/kg/IM.[4] Group 3 (DF): rats were injected DF at a dose of 10 mg/kg/IM.[13] Group 4 (CP + DF): rats were coadministrated CP and DF. All treatments were administered once daily for 14 sequential days.

2.3 | Samples collection and processing

After completion of the experiment, rats were euthanized under inhalation anesthesia using isoflurane at a dose rate of 0.6 ml/L of chamber volume.[19] Blood samples were collected forthwith from the caudal vena cava at 25°C without the addition of an anticoagulant. Following centrifugation at 3000 rpm for 10 min, sera were congregated and kept at −20°C until further use for biochemical tests. Liver, kidney, and testes tissue samples were harvested during the necropsy and perfused in ice-cold phosphate buffer saline (PBS). Each tissue specimen was split into two portions; one portion was preserved in a 10% buffered formalin for further histological and immunohistochemical assessments. The other portion was processed as mentioned later for oxidative stress biomarkers' evaluation.

2.4 | Biochemical assay

Sera were utilized for estimation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) enzymatic activities, urea, creatinine, total protein, albumin, cholesterol, triglycerides, high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) concentrations using diagnostic kits purchased from Laboratory Biodiagnostics Co. In addition, the serum testosterone level was determined using an enzyme-linked immunosorbent assay kit (Immunometrics Ltd.). All analyses were performed according to the manufacturers' instructions.

2.5 | Tissue oxidative biomarkers assay

After harvesting the tissues, each sample was rinsed with heparinized physiological buffer saline (100 mM Na_2HPO_4/NaH_2PO_4, 0.16 mg/ml heparin, pH 7.4) to get rid of RBCs and clot residues. One gram of each tissue sample was homogenized in 5 ml of cold buffer (50 mM potassium phosphate, 1 mM of ethylenediaminetetraacetic acid [EDTA], pH 7.5) utilizing a sonic homogenizer. All set homogenates were centrifuged at 4000 rpm/20 min using a cooling centrifuge then the supernatant was preserved at −20°C. Antioxidant enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) and the lipid peroxidation marker (malondialdehyde [MDA]) level were evaluated according to the manufacturer guide note (Laboratory Biodiagnostic Co.).

2.6 | Histoarchitecture and immunohistochemical assessment

Tissue specimens were collected from the liver, kidney, and testes, then fixed in 10% formalin for 24 h and dehydrated in sequential increasing concentrations of ethanol, cleared in xylene, and processed by the usual paraffin-embedding procedure. Paraffin blocks were sectioned at 5 μm thickness, deparaffinized, and ultimately stained by hematoxylin and eosin (H&E) for inspection by bright field microscopy.[20]

For immunohistochemical assessment, tissue sections were dewaxed and dehydrated in consecutive graded ethyl alcohol solutions (100%, 90%, 80%, and 70%). Retrieval of antigen by inundation in EDTA solution, pH 8 was done. Following this, the block of endogenous peroxidases by utilizing a 3% H_2O_2 solution in methanol for 5 min and then washed for three times, 5 min each, in PBS. Next, the slide was blocked in BSA (5%) for 20 min, then incubated for 1 h with primary-monoclonal antibody anti-caspase 3 (Santa Cruz Biotechnology Inc., 1:100 dilution) at 37°C. Following this, the slide was washed up with PBS three times and incubated with anti-mouse IgG secondary antibodies (Envision system labeled polymer reagent; Dako, 1:1000 dilution) till 45 min at 37°C. Ultimately, a brown stain was evident with 3,3-diaminobenzidine tetrahydrochloride (DAB; Dako), and counterstained with hematoxylin.

According to Meyerholz and Beck,[21] the ordinal scoring method was used for grading the alterations in each histological parameter in the liver, kidney, and testes. Each parameter was given a score from 0 to 4 where 0 was normal, 1 was less than 25% injury, 2 was 25%–50% injury, 3 was 5%–75% injury, and 4 was greater than 75% injury. However, the immunohistochemical expressions of caspase 3 in all examined tissues were scored as outlined by Gad et al.[22]. An intensity score (IS), 0–4 representing no staining to very strong staining, respectively, was scored to the examined cells. In addition, a proportional score (PS), 0–5 for no positive cells to greater than 65% positive cells, respectively, was recorded. The total score (TS) was calculated by the addition of IS to PS then scored at 1–3, 4–6, and 7–9 representing weak, moderate, and strong grades, respectively.

For both histological and immunohistochemical scoring, six random fields at 400X were checked blindly using the Leica DM3000 imaging system.

2.7 | Data analysis

Data visualization and statistical analyses were achieved using GraphPad Prism software version 5.0 (GraphPad Software). The significant variations among multiple groups comparisons were analyzed by one-way analysis of variance and Kruskal–Wallis test

for parametric and nonparametric values, respectively. Bonferroni test was used as a post-hoc. All values are expressed as mean ± SE and deemed significant at $p \leq 0.05$. A principal coordinate analysis (PCoA) of biochemical and antioxidant parameters was performed on all samples. The significant difference between groups was analyzed using nonparametric multivariate analysis of variance (PERMANOVA), and Bonferroni corrected p values in PAST version 3.13.

3 | RESULTS

3.1 | Biochemical parameter assay

As explicated in Figure 1, CP and/or DF treatment brought about liver, kidney, and testicular damage, that was indicated by a remarkably high level of their parameters in serum, encompassing AST, ALT, ALP, creatinine, and urea, while a noticeable decrease in albumin, total protein, and testosterone concentrations compared to control rats. Adding to this, we observed a marked accretion in serum levels of cholesterol, triglycerides, and LDL-C accompanied by a marked decrease in HDL-C (Figure 2). These observations suggest that CP or DF exposure disrupted lipid metabolism. Remarkably, there was noteworthy perturbation in the hepatic, renal, and testicular functions as well as lipid metabolism if CP and DF were coadministrated when matched to the sole treatment.

3.2 | Oxidative/antioxidative biomarkers assay

Data of antioxidant enzyme activities (SOD and CAT) as well oxidative stress molecule (MDA) are illustrated in Figure 3. Drastic elevation of MDA values with a marked decline in SOD and CAT activities as a response to CP or DF exposure in hepatic, renal, and testicular tissues ($p \leq 0.05$), when compared to control rats, was observed and confirmed the existence of oxidative stress. Interestingly, combined exposure to both remedies could evidently cause more substantial oxidative hurt in the liver, kidney, and tests than their individual exposure.

3.3 | Principal coordinate analysis

PCoA data indicated that the different studied groups were obviously distanced along the PC1 axis, accounting for the majority (88.42%) of the difference between groups. In PCoA, MDA content in all studied tissues, testosterone, cholesterol, triglycerides, and ALP were the prominent components that shifted the CP + DF cluster clearly from other treated groups, as illustrated in Figure 8.

3.4 | Histoarchitecture examination

To confirm the formerly obtained findings, the histological alterations were assessed in the liver, kidney, and testes subsequent to CP and/or

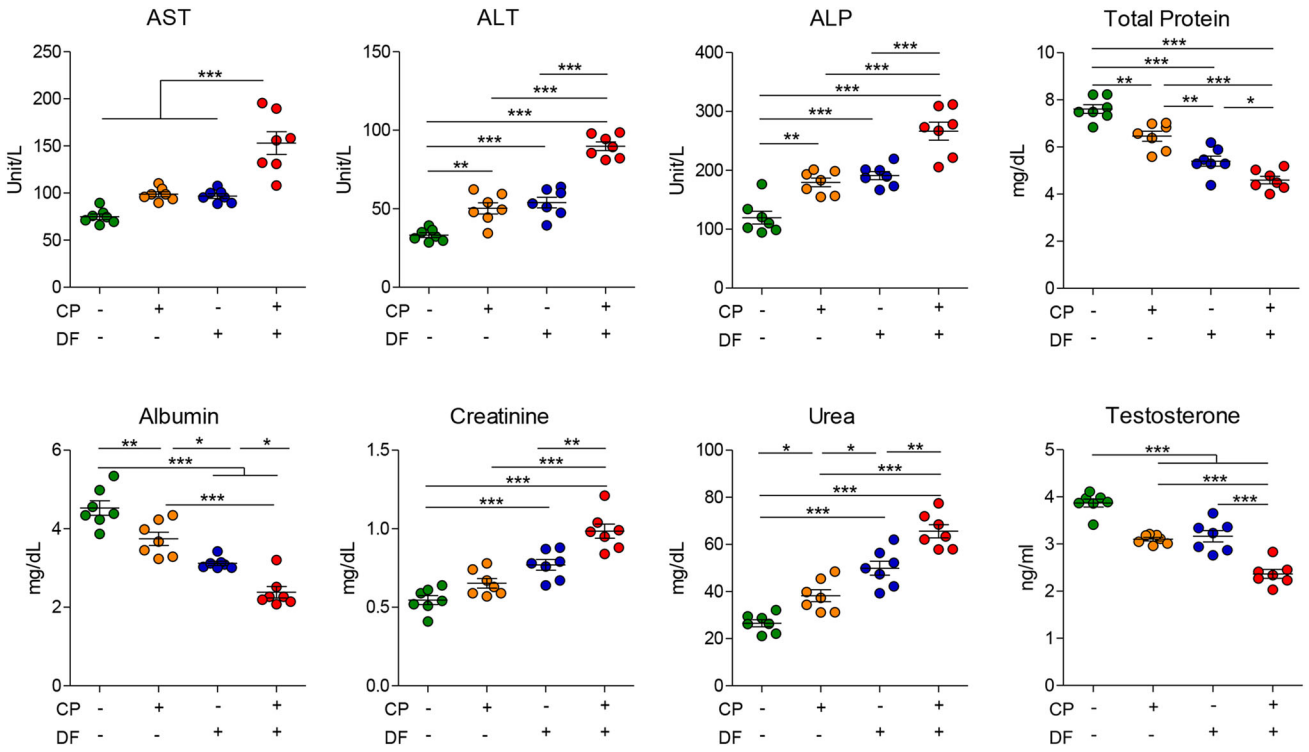


FIGURE 1 Changes in serum biochemical parameters and testosterone level after treatment with CP and/or DF. All values are expressed as the mean ± SE ($n = 7$). ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CP, cefepime; DF, diclofenac; T. protein, total protein. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$

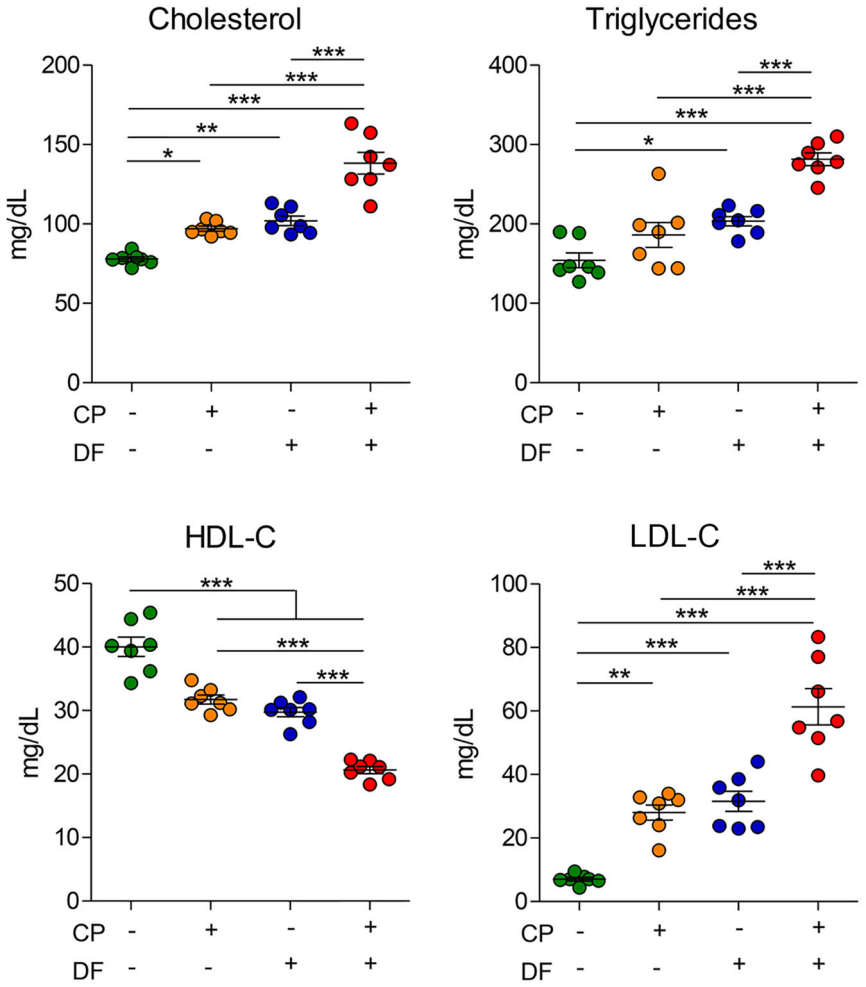


FIGURE 2 Changes in the lipid profile after treatment with CP and/or DF. All values are expressed as the mean ± SE ($n = 7$). CP, cefepime; DF, diclofenac; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$

DF treatment. As regards the liver specimen, (control) it displayed normal features of hepatic tissue; the central vein surrounded by normally arranged cord-like hepatocytes, normal sinusoids, and portal area (Figure 4A,B). Contrariwise, CP-treated tissue exhibited disorders of hepatic architectures represented by congestion of central veins and portal vessels, marked dilatation of sinusoids with stasis of red blood corpuscles (Figure 4C,D). However, rats exposed to DF also depicted congested distorted central vein and sinusoids, marked degeneration of hepatocytes (highly eosinophilic hepatocytes) with pyknosis of nuclei, bile duct proliferation, and congestion of portal vessels (Figure 4E,F). Both CP and DF treatments induced Kupffer cells hyperplasia (Figure 4C–F). Evidently, concurrent exposure to both drugs (CP + DF) showed higher significant hepatotoxicity among groups which were illustrated by marked dilatation and congestion of central vein, portal vessels, and sinusoids, focal lymphocytic aggregation, and massive infiltration of fat vacuoles inside the hepatocytes indicating severe hurt taken place (Figure 4G,H). Kupffer cell proliferation (hyperplasia) is a common finding among all groups except for the control. Scoring of histological alteration induced by CP and/or DF in the liver is recorded in Table 1.

Moreover, when we examined the renal tissue, the control group exhibited normal architectural patterns with no identifiable alterations (normal glomeruli, tubular epithelia, and interstitium), as shown in Figure 5A. Contrary to the control, the CP-treatment bred marked changes in renal histology. Congestion and dilatation of glomerular tufts, cortical blood vessels, and intertubular blood capillaries, cystic dilatation of some cortical renal tubules with the shedding of their epithelial lining together with eosinophilic debris in their lumina were observed (Figure 5B). Additionally, in the DF group, we observed atrophy of some glomeruli, desquamation of tubular epithelium in the cortex and the cortico-medullary connection, dilatation of renal tubules with intratubular eosinophilic secretion, extensive congestion of glomeruli and intertubular blood capillaries (Figure 5C). Interestingly, when CP was coadministrated with DF, the kidney tissue exhibited drastic perturbation than their individual exposure, evidenced by extensive cystic dilatation of the renal tubules, more intratubular eosinophilic renal casts in their lumen, and severe congestion of the glomerular tufts (Figure 5D). Scoring of histological alteration induced by CP and/or DF in the kidney is done in Table 2.

Next, the histological changes of combined treatment of CP and DF were assessed in testicular tissue. As revealed in (control), they had no alteration, there were well-distributed seminiferous tubules with the presence of complete spermatogenic series in their lumen. Sertoli cell and interstitial Leydig cell were normal (Figure 6A). With respect to CP treatment, we observed exfoliation of seminiferous tubules lining the

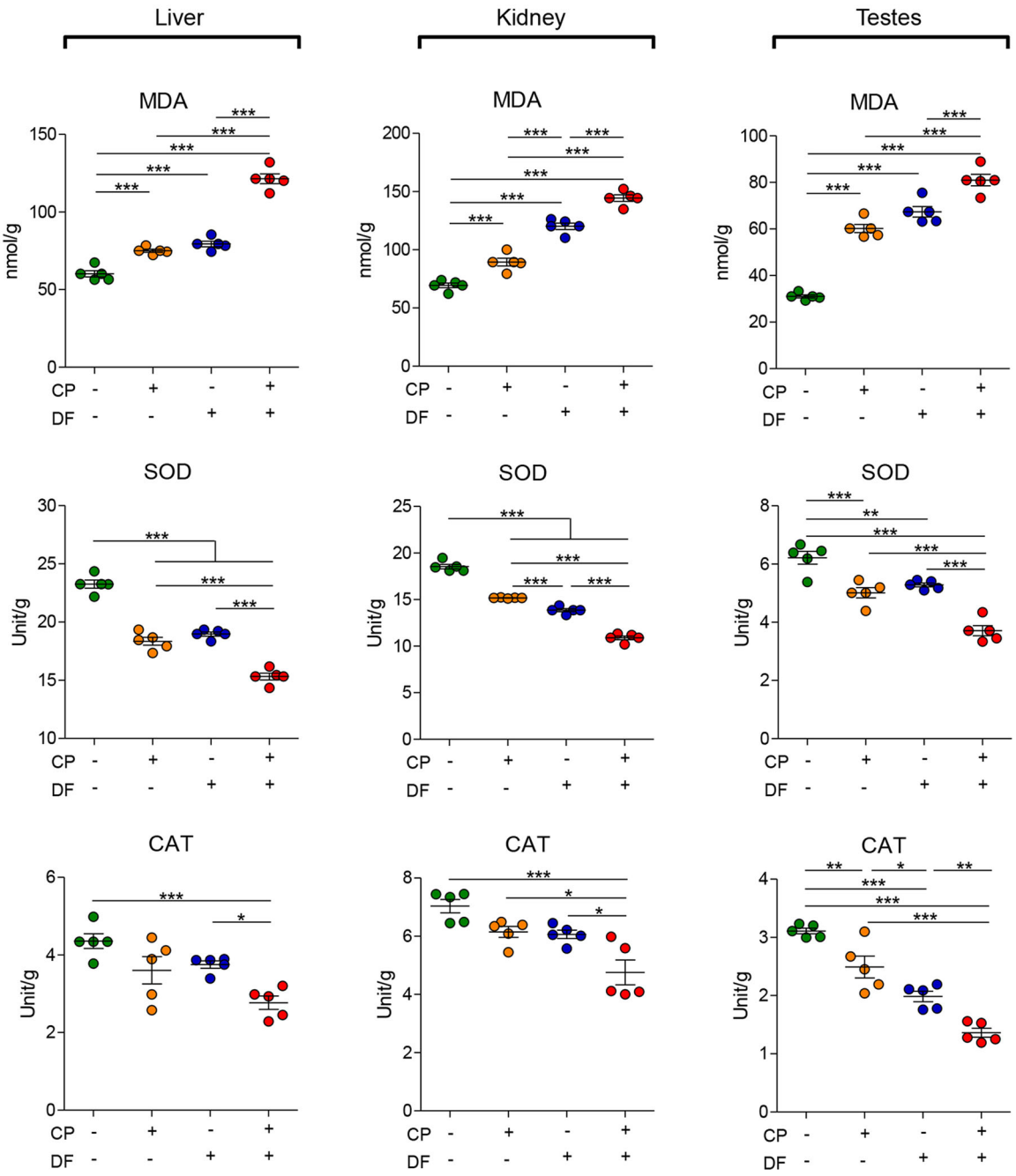


FIGURE 3 Changes in oxidative/antioxidative status after treatment with CP and/or DF. All values are expressed as the mean ± SE ($n = 7$). CP, cefepime; DF, diclofenac; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$

epithelium with reduced spermatogenic cells layer, thickened and corrugated basement membranes as well as vacuolated spermatogonia. Congestion of the interstitial blood capillaries was also detected (Figure 6B,C). However in the DF-treated group, disarranged degenerated seminiferous tubules with vacuolated spermatogonia, detachment of spermatogenic epithelium, reduction of spermatogenic cells layer, marked interstitial widening with congestion of blood capillaries were recorded (Figure 6,E). Remarkably, combined exposure to CP and DF causes augmentation of former degenerative changes of the seminiferous tubules with interstitial edema (Figure 6F). Intraluminal exfoliated cells were identified in all groups except the control (Figure 6B–F). Scoring of histological alteration induced by CP and/or DF in the testes is done in Table 3.

Expectedly, these histological findings vividly mirrored the obtained biochemical data.

3.5 | Immunohistochemical outcomes

Changes in caspase 3 expressions in the cytoplasm and/or nuclei of liver, kidney, and testes tissues are illustrated in Figure 7. CP or

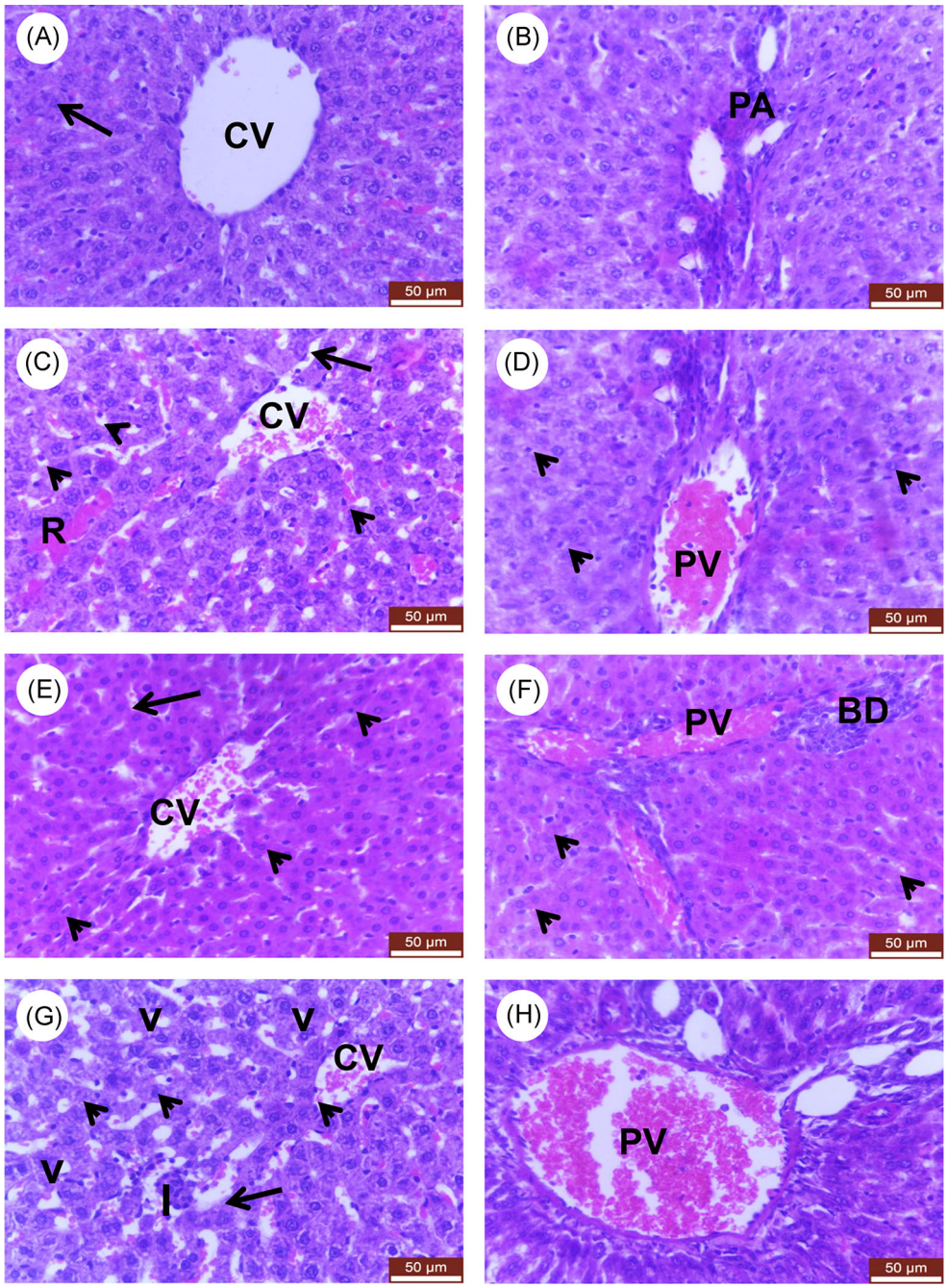


FIGURE 4 Liver histoarchitecture after treatment with CP and/or DF. (A) and (B) Control saline group, displays the normal central vein surrounded by normally arranged cord-like hepatocytes, normal sinusoid, and portal area. (C) and (D) CP-treated rat tissue section shows congestion of central veins, marked dilatation of sinusoids with stasis of red blood corpuscles and hyperplasia of Kupffer cells (C), congestion of portal vessels (D). (E) and (F) DF-treated rat tissue section shows congestion of central veins and dilatation of sinusoids, highly eosinophilic hepatocytes, Kupffer cell hyperplasia (E), bile duct proliferation, and congestion of portal vessels (F). (G) and (H) CP + DF-treated rat tissue section shows severe dilatation of sinusoids, infiltration of fat vacuoles inside the hepatocytes, focal inflammatory cells aggregation, congestion of central vein, Kupffer cell proliferation (G), severe dilatation, and congestion of portal vessels (H). (Bars = 50 µm). CP, cefepime; DF, diclofenac; CV, central vein; arrow, sinusoids; PA, portal area; arrowhead, Kupffer cells; R, RBCs stagnation; BD, bile duct; V, fat vacuoles; I, Inflammatory cells

TABLE 1 Ordinal scoring of histological alteration induced by CP and/or DF in the liver

Parameters	Experimental groups			
	Control	CP	DF	CP + DF
Congestion of central vein	0.0 ± 0.0^{b}	2.56 ± 0.06^{a}	2.56 ± 0.12^{a}	2.60 ± 0.0^{a}
Sinusoidal dilatation	0.0 ± 0.0^{d}	2.73 ± 0.06^{b}	0.70 ± 0.05^{c}	3.67 ± 0.07^{a}
RBCs stasis in sinusoids	0.0 ± 0.0^{b}	3.46 ± 0.17^{a}	0.0 ± 0.0^{b}	3.20 ± 0.11^{a}
Kupffer cells proliferation	0.0 ± 0.0^{b}	2.46 ± 0.06^{a}	2.40 ± 0.11^{a}	2.53 ± 0.06^{a}
High eosinophilic hepatocytes	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	3.60 ± 0.11^{a}	0.0 ± 0.0^{b}
Fat infiltration	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	2.67 ± 0.13^{a}
Congestion of portal vessels	0.0 ± 0.0^{b}	3.33 ± 0.06^{a}	3.06 ± 0.06^{a}	3.66 ± 0.13^{a}
Dilatation of portal vessels	0.0 ± 0.0^{d}	1.60 ± 0.11^{b}	0.66 ± 0.06^{c}	3.20 ± 0.12^{a}
Bile duct proliferation	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	2.40 ± 0.0^{a}	0.0 ± 0.0^{b}
Inflammatory cell infiltration	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.66 ± 0.08^{a}

Note: All values are expressed as the mean ± SE. Superscript letters within the same rows were significant ($p \leq 0.05$).

Abbreviations: CP cefepime; DF diclofenac.

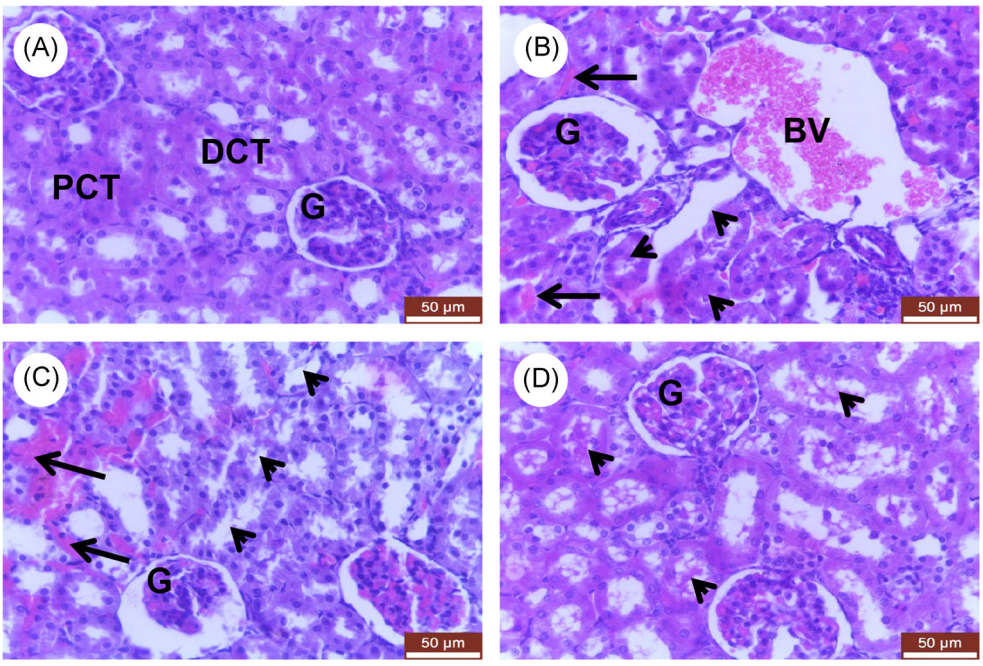


FIGURE 5 Kidney histoarchitecture after treatment with CP and/or DF. (A) Control saline-treated rat shows normal architectural patterns (normal glomeruli, proximal and distal convoluted renal tubules, and interstitium) with no identifiable alterations. (B) CP-treated rat tissue section shows, congestion of glomerular tufts, dilatation, and congestion of cortical blood vessels and intertubular blood capillaries, eosinophilic debris in the lumina of some renal tubules. (C) DF-treated rat tissue section shows atrophy of some glomeruli, dilatation of renal tubules with intratubular eosinophilic secretion, and extensive congestion of intertubular blood capillaries and glomeruli. (D) CP + DF-treated rat tissue section exhibits severe congestion of the glomerular tufts, cystic dilatation of the renal tubules, and more intratubular eosinophilic secretions. (Bars = 50 µm). BV, blood vessels; CP, cefepime; DCT, distal convoluted tubules; DF, diclofenac; G, glomeruli; PCT, proximal convoluted tubules; arrow, intertubular capillaries; arrowhead, tubular eosinophilic debris/secretion

DF noteworthily showed significant moderate expressions of caspase 3 in hepatic, renal, and testicular tissues in comparison to weak expressions in the control tissues. Moreover, concurrent treatment with CD and DF drastically aggravated the apoptotic process confirmed by significant strong upregulation of the caspase 3 compared to their single regimen. Scoring of caspase 3 expressions induced by CP and/or DF in liver, kidney, and testes is done in Table 4.

TABLE 2 Ordinal scoring of histological alteration induced by CP and/or DF in kidney

Parameters	Experimental groups			
	Control	CP	DF	CP + DF
Glomerular congestion	0.0 ± 0.0^{c}	2.86 ± 0.03^{b}	2.76 ± 0.03^{b}	3.70 ± 0.05^{a}
Glomerular atrophy	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.80 ± 0.05^{a}	0.0 ± 0.0^{b}
Congestion of blood vessels	0.0 ± 0.0^{b}	2.33 ± 0.13^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
Congestion of intertubular capillaries	0.0 ± 0.0^{c}	2.73 ± 0.06^{b}	3.10 ± 0.05^{a}	0.0 ± 0.0^{c}
Tubular dilatation	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	2.36 ± 0.08^{a}	2.80 ± 0.05^{a}
Tubular debris	0.0 ± 0.0^{c}	0.60 ± 0.05^{b}	0.76 ± 0.03^{b}	2.80 ± 0.05^{a}

Note: All values are expressed as the mean ± SE. Superscript letters within the same rows were significant ($p \leq 0.05$).

Abbreviations: CP cefepime; DF diclofenac.

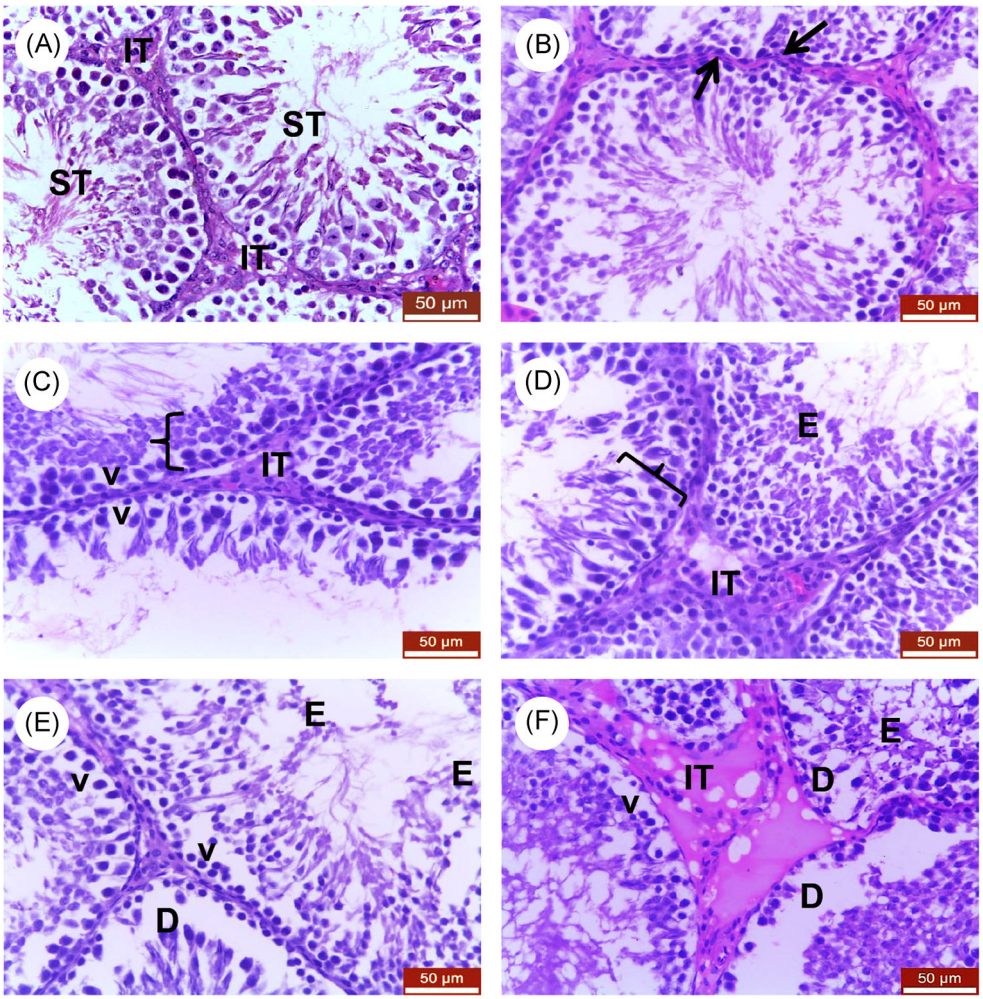


FIGURE 6 Testes histoarchitecture after treatment with CP and/or DF. (A) Control testes show no alteration, well-distributed seminiferous tubules with the presence of complete spermatogenic series in their lumen. Sertoli cell and interstitial Leydig cell are normal. (B) and (C) CP-treated rat tissue section shows thickened and corrugated basement membranes of seminiferous tubules (B), intraluminal exfoliated cells, reduced spermatogenic cells layer, vacuolated spermatogonia as well as congestion of the interstitial capillaries (C). (D) and (E) DF-treated rat tissue section shows marked interstitial widening with congestion of blood capillaries, intraluminal exfoliated cells in addition to the reduction of spermatogenic cells layer (D), degeneration of some seminiferous tubules, detached spermatogenic epithelium, and vacuolated spermatogonia (E). (F) CP + DF-treated rat tissue section shows severe degeneration of the seminiferous tubules with intraluminal exfoliated cells, vacuolated spermatogonia, and interstitial edema. (Bars = 50 µm). CP, cefepime; DF, diclofenac; ST, seminiferous tubules; IT, interstitium; arrow, basement membrane; right brace, spermatogenic cells layers; V, vacuolated cells; D, degenerated and detached spermatogenic epithelium; E, exfoliated cells

TABLE 3 Ordinal scoring of histological alteration induced by CP and/or DF in testes

Parameters	Experimental groups			
	Control	CP	DF	CP + DF
Corrugated basement membranes	0.0 ± 0.0[b]	2.60 ± 0.11[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
Tubular degeneration	0.0 ± 0.0[d]	0.76 ± 0.08[c]	2.60 ± 0.11[b]	3.76 ± 0.08[a]
Spermatogenic detachment	0.0 ± 0.0[b]	0.0 ± 0.0[b]	2.40 ± 0.11[a]	2.73 ± 0.06[a]
Reduced cells layers	0.0 ± 0.0[b]	2.20 ± 0.11[a]	2.36 ± 0.08[a]	0.26 ± 0.03[a]
Vacuolated spermatogonia	0.0 ± 0.0[c]	1.60 ± 0.11[b]	2.73 ± 0.08[a]	2.66 ± 0.03[a]
Intraluminal exfoliated cells	0.0 ± 0.0[b]	2.26 ± 0.06[a]	2.20 ± 0.11[a]	2.66 ± 0.17[a]
Congestion of interstitial capillaries	0.0 ± 0.0[d]	1.70 ± 0.05[b]	2.60 ± 0.11[a]	0.3 ± 0.05[c]
Interstitial widening	0.0 ± 0.0[c]	0.0 ± 0.0[c]	2.76 ± 0.08[b]	3.80 ± 0.0[a]
Interstitial edema	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.33 ± 0.06[b]	3.83 ± 0.03[a]

Note: All values are expressed as the mean ± SE. Superscript letters within the same rows were significant ($p \leq 0.05$).

Abbreviations: CP cefepime; DF diclofenac.

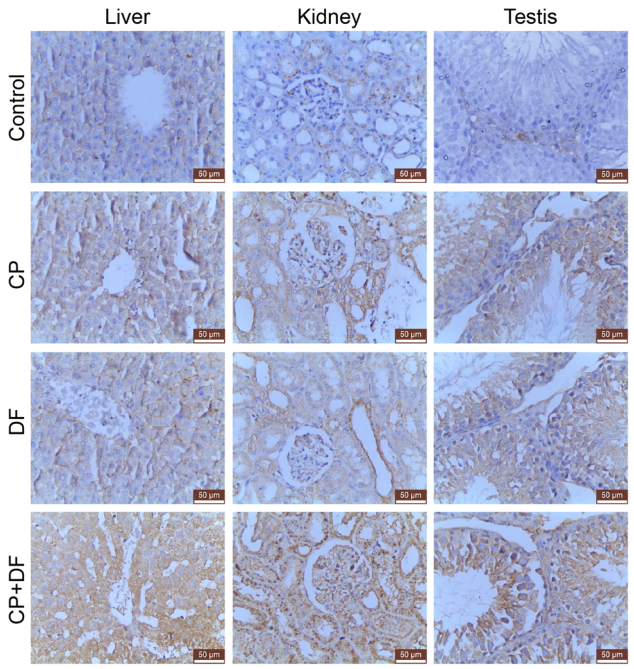


FIGURE 7 Effects of CP and/or DF on the caspases 3 expression (liver, kidney, and testes) Control saline groups display weak cytoplasmic expression of caspases 3. CP or DF-treated rats elucidated cytoplasmic moderate expression of caspase 3. CP + DF treated rats show strong cytoplasmic caspase 3 positive areas. Brown stain indicates the caspase 3 positive area. (Bars = 50 μm).
CP, cefepime; DF, diclofenac

4 | DISCUSSION

It is well known that antibiotics are commonly prescribed with NSAIDs in many comorbid conditions to combat pathogens and associated inflammatory responses. Despite their potential therapeutic index, growing evidence has shown the adverse effects of each of them. In the same line, the concurrent use of CP and DF is confounding, as the coadministration of both drugs might enhance their side effects as expounded in this study.

There is substantial evidence implicating that oxidative stress and mitochondrial disruption are common toxic mechanisms associated with CP or DF-prompted tissue injuries.[5,23] Oxidative distress is known to occur when the generated ROS (superoxide anion, $O_2^{\bullet -}$; hydrogen peroxide, H_2O_2; and hydroxyl radical, $OH^{\bullet}$) overrides the cellular antioxidant competence. That causes harm to the cell via triggering a cascade of complex biological proceedings such as peroxidation of lipid (LPO), uncoupling of oxidative phosphorylation, depletion of ATP, mitochondrial disruption, protein oxidation, DNA damage, and ultimately apoptotic cell death.[5,24]

Convincingly, the current work elucidates that ROS contributed to the toxic consequences of CP and/or DF treatment, which is notable for noteworthy declines in SOD and CAT enzymatic activities in hepatic, renal, and testicular tissues. SOD is an endogenous enzyme that represents the first line of enzymatic defense in mitochondria that dismutates the generated $O_2^{\bullet -}$ to O_2 and H_2O_2, which consequently quenches oxidative damage.[25] Additionally, CAT accelerates the decomposition of H_2O_2 into water and O_2. In the case of CAT exhaustion, abundant quantities of $OH^{\bullet}$ (the most mischievous reactive radical) are generated from H_2O_2 by Fenton's reaction, which directly attacks the membrane lipid resulting in LPO and enhanced MDA production.[26] Alarmingly, MDA can also speed up other key subcellular molecules (DNA and proteins), making matters worse. Similar to this assertion, the current study revealed significant increments in MDA concentrations affirming the incidence of membrane damage. Losing of membrane integrity and increased permeability possibly contributed to the leakage of hepatic intracellular enzymes (AST and ALT) and a membrane-bounded enzyme (ALP) into the bloodstream, increasing their levels in the serum which confirms hepatic dysfunction.[7,9] These findings are congruent with that obtained by a preceding study of Adeyemi and Olayaki[13] who used the DF as a model for induction of oxidative damage in liver tissue. The current study also noted that CP caused hepatic injury,

TABLE 4 Caspase 3 immunohistochemical expressions induced by CP and/or DF in liver, kidney, and testes

Groups	Liver			Kidney			Testes		
	PS	IS	TS	PS	IS	TS	PS	IS	TS
Control	2.13 ± 0.0	0.66 ± 0.03	2.80 ± 0.05^{c}	1.70 ± 0.05	0.80 ± 0.05	2.50 ± 0.11^{c}	1.16 ± 0.03	0.50 ± 0.05	1.66 ± 0.06^{c}
CP	3.80 ± 0.0	2.53 ± 0.08	6.33 ± 0.08^{b}	3.80 ± 0.0	2.86 ± 0.08	6.66 ± 0.08^{b}	3.80 ± 0.03	2.93 ± 0.03	6.73 ± 0.03^{b}
DF	3.63 ± 0.03	2.40 ± 0.05	6.03 ± 0.06^{b}	3.80 ± 0.0	2.40 ± 0.0	6.20 ± 0.0^{b}	3.66 ± 0.03	2.73 ± 0.03	6.40 ± 0.10^{b}
CP+DF	4.90 ± 0.0	3.60 ± 0.0	8.50 ± 0.0a	4.86 ± 0.0	3.86 ± 0.03	8.73 ± 0.03^{a}	4.50 ± 0.03	3.80 ± 0.05	8.26 ± 0.12^{a}

Note: All values are expressed as the mean ± SE. Superscript letters within the columns of TS for each organ were significant ($p \leq 0.05$).
Abbreviations: CP, cefepime; DF, diclofenac; IS, intensity scores; PS, proportional scores; TS, total scores.

elucidated by histological findings, but when concurrently used with DF, higher significant hepatic damage has occurred compared to other groups. These data are in agreement with the findings of Balubaid[27] that evinced liver injury in various gestational periods in rats intoxicated by CP. Also, in conformity with those reported by Rawlani[28] who demonstrated DF caused significant toxic damage to liver cells of mice.

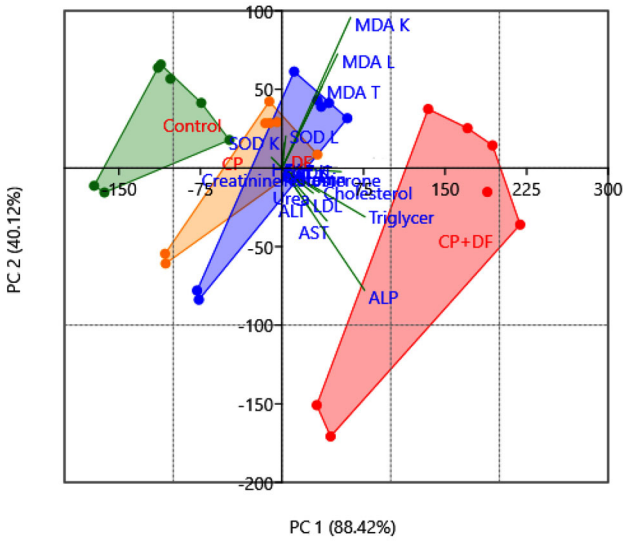
LPO was observed in the renal cell membrane elucidated in the renal histology by disintegrated cortical tubular lining epithelia along with a significant increase in serum urea and creatinine levels. These findings confirmed the existence of renal insufficiency. Importantly, as the mitochondria are more abundant in the proximal tubules, they made them more vulnerable to ROS-induced oxidative damage and apoptotic changes. These data strongly support our previous reports, which indicated the vulnerability of the cortical tubules to the oxidative damage induced by antibiotics, puromycin[29] and gentamicin[30] and NSAIDs, piroxicam[9,25] and paracetamol.[31] Therefore, it is strongly proposed that mitochondria are a potential subcellular target for CP and DF. The marked histological degenerative changes of the renal tissues that were elucidated in our result were consistent with the beforehand studies.[4,32]

DF is ascribed to suppress prostaglandins synthesis that is involved in regulating renal blood flow and glomerular filtration rate via preferential COX-I inhibition leading to lowering of the renal blood flow and increased risk of renal ischemia[32] which was reflected by some glomerular atrophy as demonstrated by our histological finding. Thus, renal ischemia could be another possible mechanism related to DF-induced nephropathy.

In an endorsement of previous studies, our study revealed that CP or DF was accompanied by substantial reductions in serum total protein and albumin concentrations.[4,13,33] These reductions might be attributed to the altered protein synthesis in hepatocytes resulted from the ROS-induced DNA and protein damage, which in turn inhibit mRNA transcription and translation processes.[33] These degenerative changes are confirmed by the histological examination (Figure 5). In addition, our and others' previous studies have documented the involvement of impaired tubular reabsorption in the increased loss of proteins in urine and reduction of their blood levels as indicated in the current study.[4,25,31] Furthermore, we noticed a disturbance in lipid metabolism inferred by enhanced serum levels of cholesterol, triglycerides, and LDL which emphasize the existence of a liver injury in response to CP or DF that was clearly obvious in our histological finding as fat infiltration in hepatocytes of CP + DF group. These data are in concordance with those reported by Adeyemi et al.,[10] who demonstrated significant elevations in cholesterol and LDL-C level in DF-intoxicated rats. Besides this, our data are in agreement with the findings of Al-Jowari[34] that demonstrated disrupted lipid profiles in rabbits intoxicated by cephalexin (other cephalosporins). The potentiated damaging effects of both CP and DF on liver cells have aggressively reflected on the lipid profile compared to their single regimen.

Intriguingly, coexposure to both remedies exhibited notable disruption of the male reproductive function indicated by altered testicular histology and decreased serum testosterone level. A growing body of evidence supposes that oxidative stress is the main mechanism that underlies testicular damage and endocrine disruption[10,21,33,35] ROS may alter the Sertoli cell microenvironment through disruption of protein synthesis required for germ cell differentiation.[11] It has been reported that the LPO of the mitochondrial membrane in Leydig cells (the primary site of testosterone biosynthesis) inhibits the trafficking of cholesterol to the inner mitochondrial membrane; thereby, the process of steroidogenesis was suppressed.[36,37] On the other hand, ROS can cross the blood–brain barrier and alter the release of gonadotrophic hormones from the hypothalamus, including testosterone.[10] These mechanisms may explain the reduced testosterone levels in our observation. Notably, cotreatment with CP and DF exhibited a more observable disruption of the male reproductive function. These findings are confirmed by our histoarchitecture alteration of seminiferous tubules and testicular interstitium and in the same lines as those obtained by the former studies on DF[35] and CP[7] treated rats.

Apoptosis is an orchestrated cell death that is controlled by several signals and metabolic proceedings. ROS are known to overwhelm the mitochondrial function and their membrane potential, provoking the release of cytochrome c into the cytosol and consequently, the caspase cascade is activated and eventually cell death.[38] The current study confirms the above-mentioned mechanism, as cellular apoptosis was significantly instigated after CP and/or DF-treatment demonstrated by upregulation of caspase 3 protein expression in hepatorenal and testicular tissues. These data are in the same vein as an in-vitro study of Gómez-Lechón et al.[38] who evaluated the apoptotic impact of the DF on human and rat hepatocytes. In another study, caspase 3 was overexpressed in kidney tissue in response to DF treatment.[22,38] It was noteworthy that a significant strong expression of caspase 3 in all examined tissues was noticed in CP + DF group compared to others



	Control	CP	DF	CP+DF
Control		0.0172	0.005	0.0006
CP	0.0172		0.4164	0.0008
DF	0.005	0.4164		0.0064
CP+DF	0.0006	0.0008	0.0064	

FIGURE 8 Principal coordinate analysis of the serum biochemical and tissue oxidative stress parameters. The percent of variation explained by each principal coordinate is indicated on the axes. The points represent individual rat data from each group ($n = 7$ rats/treatment) as: control (green), CP + DF (red), DF group (blue), and CP (orange). CP, cefepime; DF, diclofenac

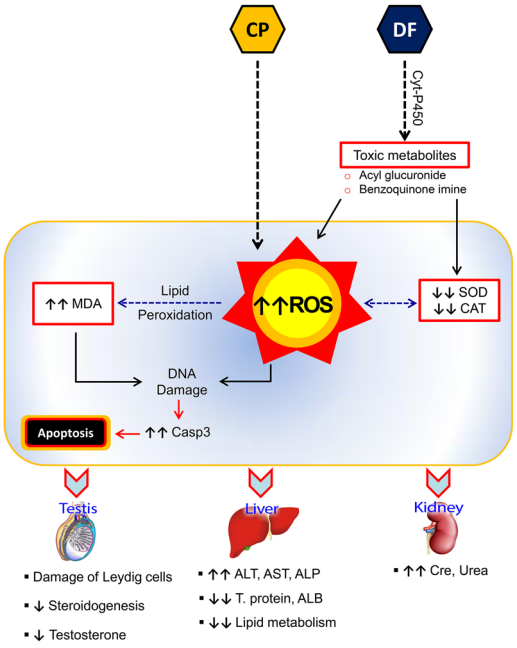


FIGURE 9 Diagrammatic scheme outlines the suggested mechanisms of potentiated hepato-renal and testicular damage after combined treatment with CP and DF. ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Casp3, activated caspase 3; CAT, catalase; CP, cefepime; Cre, creatinine; Cyto-P450, cytochrome-P450; DF, diclofenac; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase

indicating a more serious effect of CP and DF combination on hepatic, renal, and testicular tissues.

Interestingly, the applied PCoA delivers new meaningful information about the side effects of both CP and DF when given in combination compared to their individual treatments. The data obtained from PCoA showed how the combined therapy of CP and DF causes more injurious deviation in the MDA content in liver, kidney, and testicular tissues as well as in the testosterone, cholesterol, triglycerides, and ALP levels; herein, these animals can be potentially discriminated from those treated with CP or DF alone with approximate 88.42% (Figure 8). To the best of our knowledge, the PCoA is performed for the first time to determine the most influential parameters implicated in the mechanisms of CP and DF combined therapy-induced hepato-renal and testicular damage. Overall, the suggested proposed mechanisms of CP and DF-potentiated damage are outlined in Figure 9.

5 | CONCLUSION

The concurrent CP and DF treatment elicited additional extensive hepatic, renal, and testicular injury than their individual treatment. Oxidative stress and disrupted lipid metabolism were the main mechanisms implicated in this potentiated toxicity. The PCoA highlighted the role of MDA, testosterone, cholesterol, triglycerides, and ALP in discriminating the CP and DF combination among other groups. Based on this study, prescribing CP with DF should be done with greater attention and rigorous precautions to avoid their potential side effects. We also anticipate that our findings may provide important references for the clinical treatment of the current COVID-19 pandemic.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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