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Article

Organic osmolyte betaine mitigates the deleterious effects of Diclofenac in vivo in wistar albino rats

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Diclofenac sodium (DF) is a non-steroidal anti-inflammatory drug (NSAID) that possesses antipyretic, analgesic, antinociceptive and anti-inflammatory activities. Like other NSAIDs, DF is known to be associated with renal, cardiovascular, and gastrointestinal complications. The present study was carried out to evaluate the adverse effects of DF *in vivo* in wistar albino rats and to assess if oral administration of the organic osmolyte betaine mitigates the adverse effect of DF. Eighteen male Wistar rats were divided into three groups, one group of animals was fed orally with 20 mg/kg of DF once/day, and the other group received a combination of 20 mg/kg of DF and 30 mg/kg of betaine, once/day. Apart from the hematological and biochemical parameters, histopathological changes in the liver, lungs, brain, heart and kidney were also investigated. Histopathological alterations that were found in the liver, kidney, and lungs of DF-treated animals were found to be minimal or absent in DF + betaine-treated animals, as compared to untreated control. The results showed that betaine mitigates the adverse effects associated with DF treatment.

Keywords: Diclofenac. Osmolytes. Betaine. Wister Rats.

Abbreviations: DF – Diclofenac Sodium, NSAID – Non-Steroidal Anti-Inflammatory Drug

INTRODUCTION

Diclofenac (DF) is a widely used non-steroidal anti-inflammatory drug (NSAID) that is available over the counter in many countries. It is being commonly used as a primary treatment for inflammatory and degenerative rheumatic diseases. It is also used for treating painful conditions that arise from inflammation of non-rheumatic origin and acute attacks of gout (Abhishek, Roddy, Doherty 2017). The drug is extensively used as tablets, capsules, ointments, and intravenous solutions. Topical DF is very effective as it quickly penetrates the subdermal tissues, acting directly on the site of inflammation and pain (Singh, Roberts, 1994). In a more recent study, topical DF was shown to relieve the pain and stiffness associated with osteoarthritis (Bariguian Revel, Fayet, Hagen, 2020). The drug has been shown to exert analgesic (pain reliever), antinociceptive (pain modulator), antipyretic (pain inhibitor) and anti-inflammatory properties through various mechanisms. Similar to other NSAIDs, DF also acts as an inhibitor of COX-1 and COX-2, thereby inhibiting the synthesis of prostaglandins (Gan Tj *et al.*, 2010). Further, DF exhibits significant anti-bacterial activities, both in vitro and in vivo that has been exploited in treating urinary tract infections (Lagadinou*et al.*, 2020).

Though DF is the most widely prescribed NSAID that is being commonly used in low, middle and high-income countries, it is known to be associated with severe dose-dependent renal, cardiovascular, and gastrointestinal complications (Mcgettigan, Henry, 2013; Sostres, Gargallo, Lanas, 2013; Varga, Sabzwari, Vargova, 2017). There are various reports on the harmful effects

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of DF in animal models. For instance, high morbidity and microscopic changes in the lungs, kidney, heart, and liver were observed in DF-treated wistar strain rats (Tomic *et al*., 2008). Likewise, histological changes in liver and kidney and changes in biochemical, oxidative and hematological parameters were observed in male albino rats that had received intramuscular injection of DF (13.5 mg/kg b.wt.) for 14 days(El-Maddawy, El-Ashmawy, 2013). However, the DF-induced toxicity has been observed to be mitigated or diminished by certain compounds such as kolaviron extracted from Garcinia kola seeds, Ajwadate extract and Spirulinafusiformis (Alabi *et al*., 2017; Aljuhani *et al*., 2019; Peter *et al*., 2017). In one of the recent study, oral administration of gallic acid was shown to reduce DF-induced liver toxicity in rats (Esmaeilzadeh *et al*., 2020). Further, the flavonoid compound Silymarin exerts protective effects against DF-induced liver toxicity in male wistar rats (Ramezannezhad, Nouri A, Heidarian, 2019).

Betaine is a highly soluble trimethyl derivative of glycine and is known to exist in zwitter ion form at neutral pH. It occurs naturally in various foods and is also synthesized in the liver due to choline metabolism. During osmotic stress, betaine is known to be accumulated in plant and animal tissues. It acts as an osmoprotectant, mainly in the liver, brain and kidney, thereby protecting the cells from osmotic stress (Kempson, Vovor-Dassu, Day, 2013). Betaine was shown to reduce the hemolysis induced by hypo osmotic stress via inhibition of RBC membrane ATPases (Moeckel *et al*., 2002). Besides functioning as an osmolyte, betaine is also act as a methyl group donor, thereby playing a role in methylation (Day, Kempson, 2016; Zhang *et al*., 1996).

Betaine exerts its anti-inflammatory effects, in vitro and in vivo via inhibition of NF-κB signaling pathway (Yi, Kim, 2012). The anti-inflammatory effects of betaine have been exploited in the treatment of nonalcoholic and alcoholic liver diseases (Kathirvel *et al*., 2010; Kharbanda *et al*., 2009). The antioxidant property of betaine is also evident from its protective effects against myocardial infarction induced in male albino rats (Ganesan *et al*., 2010). Betaine also exerts protective effects against hepatotoxicity and oxidative damage by regulating oxidative stress (Jiang *et al*., 2019; Özkoç *et al*., 2020).

In this study, we analyzed the adverse effects of DF in wistar albino rats by monitoring the biochemical and hematological parameters and observing the histological changes in the brain, heart, liver, lungs, and kidneys. Further, we sought to determine whether oral administration of betaine and DF would mitigate the adverse effects associated with DF treatment.

MATERIAL AND METHODS

Reagents

Betaine, DF, and sodium cacodylate were obtained from Sigma Chemical Co. Potassium chloride from Sisco Research Laboratories (SRL). Various other chemicals used were of analytical grade.

Animal care

18 adult male wistar albino rats, weighing 200-250 g and 18 weeks old, were used for the study. The animals were procured from a closed-chambered bred colony from the Chettinad Hospital & Research Institute (CHRI), Chennai, India. The wistar albino rats were housed in polypropylene cages (2 rats/cage), measuring 148.3 cm^2 and height 14 cm^2 (floor area/animal), with free access to food pellets and water. The standard laboratory diet was procured from Chettinad Hospital & Research Institute (CHRI), Chennai- India. All the 18 wistar albino rats were exposed to 12h light/12h dark cycle and were housed under similar environmental conditions for 4 weeks before administering the drugs so that the animals get acclimatized to the states. The experimental procedure was approved by the Institutional ethical committee, Chettinad Hospital & Research Institute (CHRI), Chennai, India (IAEC reference no. CHRI/DEAN/PROJ-006/2018). The animal studies were performed as per the committee's guidelines for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Experimental Protocol

The 18 adult male albino rats were randomly divided into three groups, each consisting of 6 rats. After

acclimatization, rats were randomly divided into three groups (6 rats each):

Group I: Rats were orally dosing administered with distilled water (1 ml/ kg b.wt.) and kept as the control group.
Group II: Rats were oral dosing with diclofenac sodium at 20 mg/kg b. wt.and dissolved in 1ml distilled water.
Group III: Rats were oral dosing with diclofenac sodium-betaine at 20+30 mg/kg b. wt. in 1 ml distilled water. Diclofenac sodium-betaine, Diclofenac sodium, and distilled water were administered for four weeks (28 days) daily. After the experimental period, animals were euthanized by administering an overdose of halothane (5 ml/kg).

*Blood collection***:** 0.5 ml of blood was collected twice from the retro-orbital plexus of rats from all the groups 24 h after the last oral administration. The primary sample was collected in potassium EDTA tubes and was used for hematological analysis. The other sample was collected in tubes without EDTA; the serum was separated and stored at -20°C until further use.

*Blood Analysis***:** Sahli's hemocytometer was used to determine the Hemoglobin (Hb) concentration, followed by the Benjamin techniques. Erythrocyte and total leukocyte counts were performed using a Double improved Neubauer hemocytometer.

*Biochemical Analysis***:** AST activities were measured according to Keiding*et al*., 1974. The enzymatic colorimetric method was used to measure the serum urea activity (Chen *et al.*, 2015). Serum globulin level was measured by deducting the value of albumin from the total protein of the sample. Serum creatinine activity was calculated at kinetic mode (Ripamonti *et al.*, 1984). The total levels of albumin were measured by the colorimetric method.

Histopathological Examination

After necropsy, the liver, lungs, brain, and kidney tissues were collected and swiftly secured in 10% formalin solution. The samples were then refined using the paraffin embedding technique that involves dehydration in increasing grades of ethyl alcohol, clearing using xylene, and embedding in melted paraffin wax at 60°C. Paraffinized blocks were cut into 5 microns sections using a microtome. The slides were stained using Hematoxylin and Eosin for further examination.

Statistical Analysis

The experimental results were subjected to the comparison of means by analysis of variance (One-Way ANOVA for parametric data) followed by Dunn's post hoc test, with a significance level of $P < 0.05$ and expressed as a mean ± standard deviation.

RESULTS AND DISCUSSION

The analgesic, antipyretic and anti-inflammatory properties of NSAIDs make them the most commonly prescribed and used therapeutic agents for treating rheumatic diseases, degenerative joint disease, inflammation, and surgical pains. DF has been shown to induce hepatotoxic and nephrotoxic effects in humans and experimental animals (Dhanvijay, Misra, Varma, 2013).

Betaine, widely distributed in plants, animals, and microorganisms, is a significant nutrient in various foods. Besides its physiological role as an osmoprotectant and a methyl group donor, betaine also exerts anti-inflammatory effects, regulates energy metabolism, and mitigates endoplasmic reticulum stress and apoptosis (Zhao *et al.*, 2018). Betaine is known to regulate protein levels in the kidney, thereby exerting anti-hyperuricemia and nephroprotective activities. Also, dose-dependent improvement in kidney function was observed in hyperuricemic mice that were orally administered with different concentrations of betaine (5, 10, 20, and 40 mg/kg) (Liu *et al.*, 2014).

TABLE I - Serum and hematological parameters of animals treated with DF and DF + betaine. Effect of oral administration of 20 mg/kg Diclofenac sodium (DF) and a combination of 20 mg/kg DF and 30 mg/kg betaine for 28 consecutive days on the Hemoglobin (Hb) concentration, Red blood cell, White blood cell, and platelet count of rats. *p≤0.05 and **p≤0.01

Parameters	Control	DF	DF + Betaine
Hemoglobin concentration (g %)	15.05 ± 0.22	14.30 ± 0.24*	15.15 ± 0.23
Red Blood Cell count (106/µl)	05.90 ± 0.18	05.51 ± 0.21*	06.07 ± 0.19
White Blood Cell count (103/µl)	09.20 ± 0.42	08.52 ± 0.35*	10.40 ± 0.36
Platelet count (lakh/µl)	04.62 ± 0.23	03.84 ± 0.25*	05.40 ± 0.21
Serum urea level (mg /dl)	34.50 ± 2.83	45.80 ± 2.45**	39.00 ± 2.31
Serum Creatinine level (mg/dl)	0.60 ± 0.05	0.57 ± 0.01	0.50 ± 0.02

Alterations in hematological parameters may indicate toxicity and physiological changes (Khan, Zafar, 2005). In the present study, a decrease in Hb, RBCs, WBCs, and platelet count in DF-treated rats suggests that DF had induced toxicity which may be characterized by the destruction of RBCs (Table I). It may indicate gastrointestinal bleeding due to the oral administration of DF (20 mg/kg b.wt). The above observation is in complete agreement with a previous study in which a significant reduction in Hb, RBC, and WBC levels was observed in adult male rats receiving an intramuscular injection of 13.5 mg/kg b.wt. DF for 14 days compared to the untreated control (El-Maddawy, El-Ashmawy, 2013). However, no significant difference in Hb, RBCs, WBCs, and platelet levels were observed in DF + betaine-treated rats compared to the untreated control (Table I). It could be an indication that betaine had counteracted the toxic effects of DF, which has been previously reported to cause gastrointestinal ulcerations in rats (Mostafa *et al.*, 2020). WBCs are essential constituents of innate immune defense and may be considered an indicator of overall health status. A decrease in WBC count in DF-treated rats may further indicate deterioration of those animals' overall health compared to the untreated control. An increase in WBC count was observed in those animals that had been administered a combination of DF and betaine. The observed decrease in WBC count in response to DF treatment follows a previous study, wherein a significant decline in WBC count was observed in mice that had received an intraperitoneal injection of 2.37 mg/kg b.wt DF for five days (Soussi *et al.*, 2019).

Determining the serum levels of urea and creatinine play a significant role in the diagnosis of kidney injury. It could be revealed that DF affected renal function as observed by a significant increase in serum urea levels in DF-treated mice as compared to the untreated control (Table I). As previously reported, the increase in urea concentration may be attributed to renal dysfunction (S, Evan Prince, 2018). In the case of animals co-treated with DF and betaine, there was no significant increase in serum urea levels, reflecting betaine's protective role in DF-induced renal dysfunction (Table I). However, no significant change in serum creatinine levels was observed between the three groups of animals (Table I).

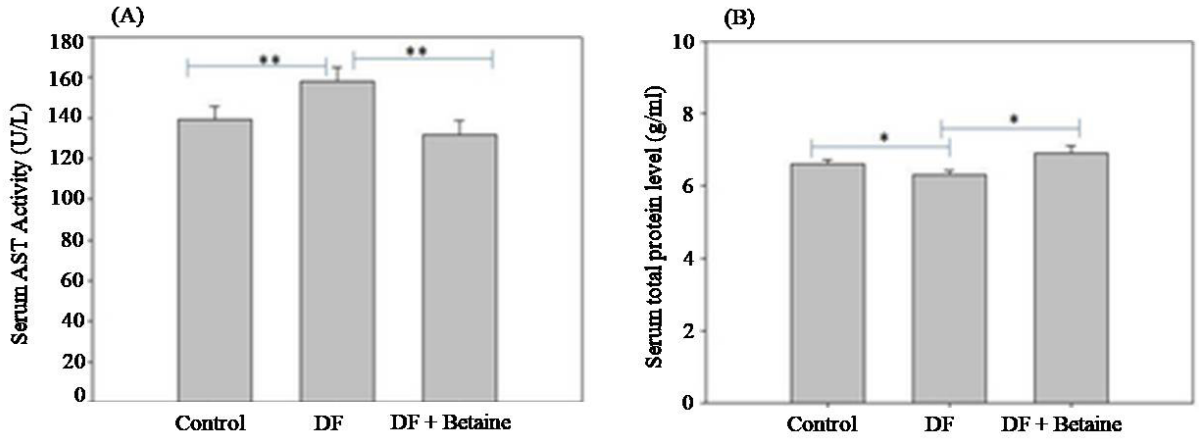

FIGURE 1 - Effects of oral administration of 20 mg/kg DF and combination of 20 mg/kg DF and 30 mg/kg betaine for 28 consecutive days on the serum (A) AST (B) total protein. *p≤0.05 and **p≤0.01 as compared to control.

The enzyme AST is present in significant concentrations in the liver, kidney, heart, and brain tissues, and the release of the enzyme into the blood circulation is considered a biomarker of damage to these tissues (Thapa, Walia, 2007). In this study, a significant increase in AST activity in DF-treated animals compared to the control animals confirmed DF-induced damage to these tissues. The serum AST activity in DF + betaine-treated animals was almost similar to that of the control animals (Figure 1A). Also, a decrease in total protein may indicate impairment in liver function. However, no significant change in the total protein content was observed in DF-treated animals and DF + betaine-treated animals compared to the control animals (Figure 1B).

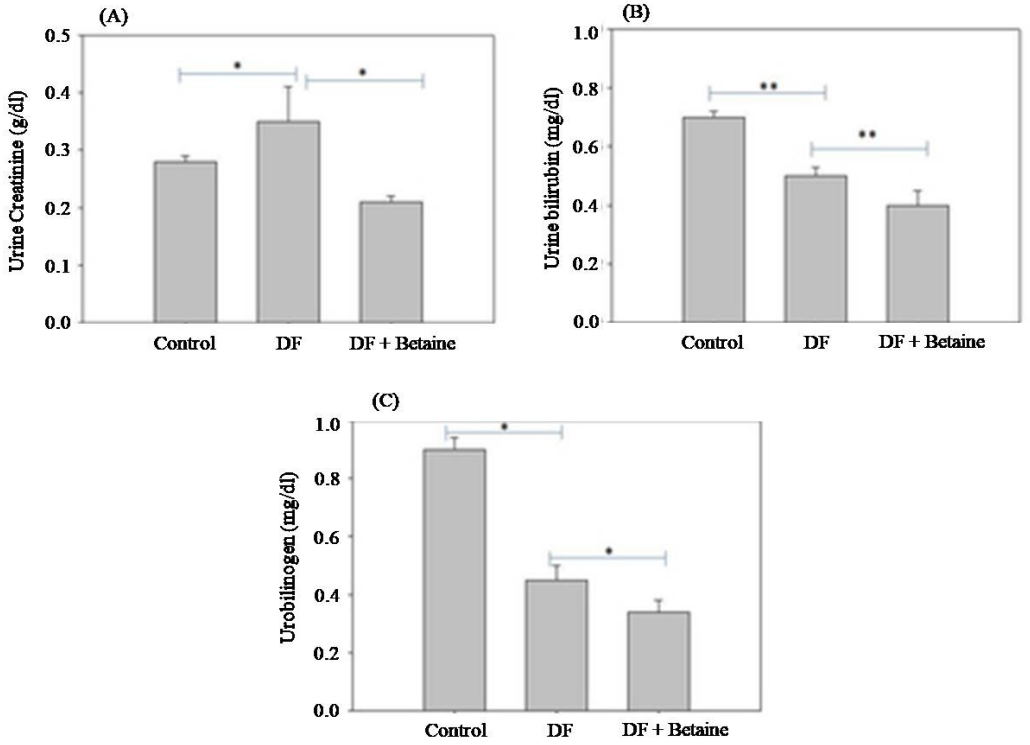

FIGURE 2 - Effects of oral administration of 20 mg/kg DF and combination of 20 mg/kg DF and 30 mg/kg betaine for 28 consecutive days on (A) Urea creatinine (g/dl) (B) Urine bilirubin (mg/dl) (C) Urobilinogen (mg/dl)levels. *p≤0.05 and **p≤0.01 as compared with control.

The changes in the creatinine level in urine are an effective biomarker of impairment of renal function due to chemical stress (Gowda *et al.*, 2010). It could be revealed that DF affected renal function as observed by a marked increase in urine creatinine levels in DF-treated mice compared to the untreated control. However, group that was co-treated with DF and betaine, the level of urine creatinine was shown to be significantly reduced (Figure 2A). A decrease in urine bilirubin and urobilinogen levels was observed in both DF alone treated groups of animals and in those administered with a combination of DF and betaine. However, the levels of bilirubin and urobilinogen were also shown to be reduced in DF + betaine-treated group (Figure 2B, C). Since the results of urine biochemistry were inconclusive, the histopathology of the liver, kidney, heart, lungs, and brain of all three groups of animals was analyzed to understand if DF induced hepatotoxic and nephrotoxic effects and to infer whether betaine counteracts the same.

DF has been shown to induce microscopic changes in the liver and kidney, indicating its dysfunction. Moreover, high mortality have also been extensively observed in animal models. For instance, histopathological alterations in the kidney and liver were observed in male wistar rats that had received an intramuscular injection of 10 mg/kg DF for 7 days (Alabi, Akomolafe, 2020). In another study, histopathological changes in hepatic and renal tissues were found in male albino rats that had been orally administered with 2.5 mg/kg DF four times per week for eight weeks (Mousa *et al.*, 2020). Likewise, the implications of betaine in protecting against liver toxicity and kidney damage have been reported in different experimental models (Heidari *et al.*, 2018). In the present study, histopathological changes observed in the liver, kidney, and lungs of animals that were administered with DF alone as compared to the experimental control group further confirm that DF treatment induced liver toxicity and damage to the kidney and lungs. Light microscope analysis of the kidneys of group I rats showed a standard histologic framework with normal proximal convoluted tubules lined by cuboidal epithelium. However, the kidney of rats treated with DF alone for four weeks showed congested glomerulonephritis with proximal tubules, focal tubular necrosis, and mild interstitial congestion with foci of lymphoplasmacytic infiltrate. In the kidneys of rats that had been administered with DF and betaine, only mild interstitial congestion and focal tubular necrosis were observed. There was no evidence of glomerulonephritis (Figure 3A).

Likewise, light microscope analysis of hepatocytes of control animals showed normal histology. The hepatocytes of animals that had been administered with DF for four weeks showed sinusoidal dilatation with congestion, necrosis, and mild periportal chronic inflammation, while only congestion was observed in livers of rats that of rats that had been orally administered with a combination of DF and betaine. The hepatic tissues appeared almost normal in the liver of group 3 animals (Figure 3B).

As shown in Figure 3C, the lungs of rats from group I showed standard histological architecture with no specific changes. However, the lungs of rats from group 2 showed emphysematous dilatation of alveoli with marked interstitial congestion and foci of dense lymphocytic infiltrate and foamy histiocytic collection (Figure 3C). In contrast, the lungs of rats from group 3 showed emphysematous dilatation of alveoli with mild interstitial congestion, and few lymphocytic infiltrates (Figure 3C).

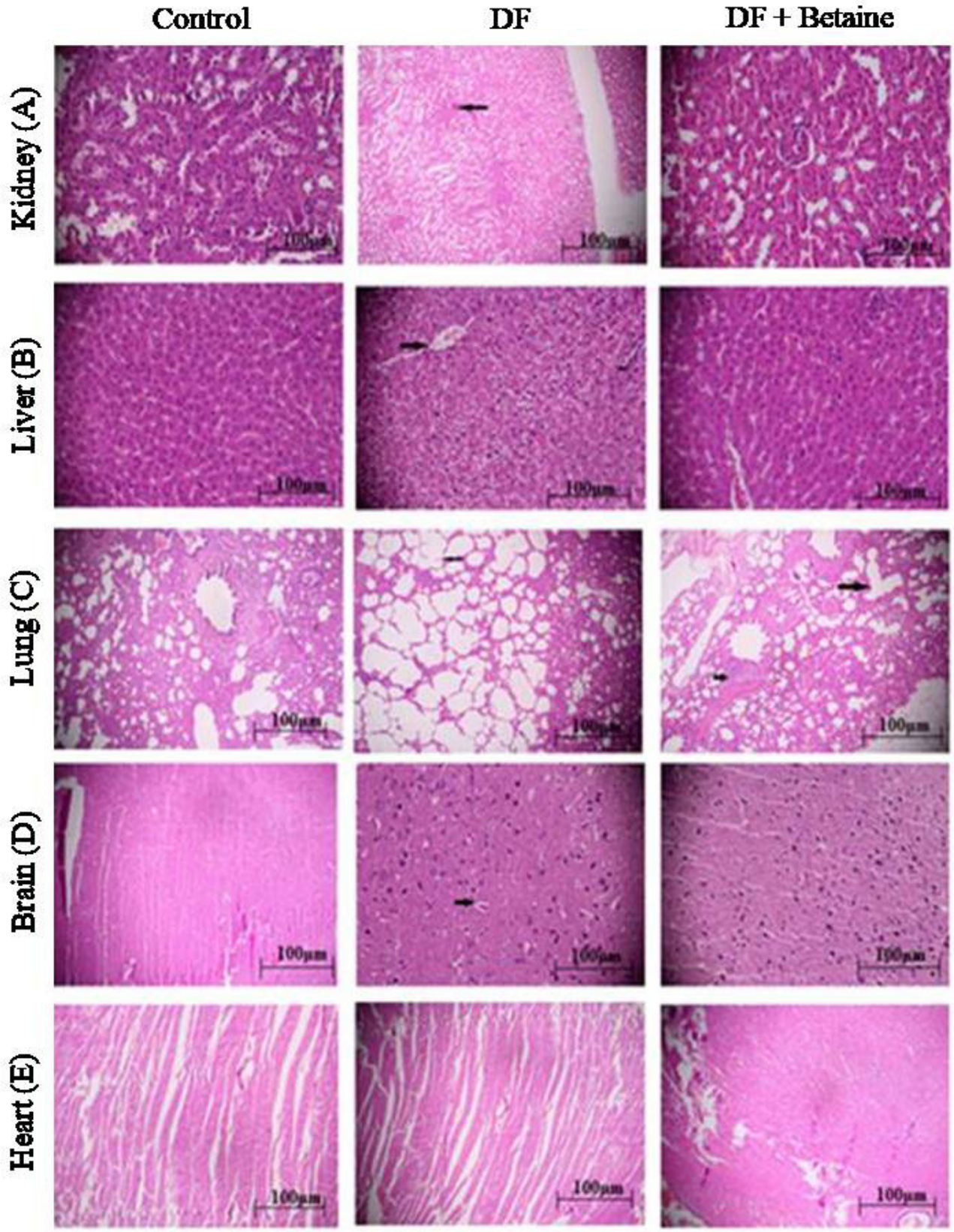

FIGURE 3 - Histopathological examinations. Photomicrograph of the rat kidney (A), liver (B), lung (C), brain(D), and heart (E) stained with hematoxylin and eosin of control rats, rats treated with 20 mg/kg DF, and rats treated with a combination of 20 mg/kg DF and 30 mg/kg betaine.

As shown in Figure 3D, normal histological architecture was observed in the brain of rats from group 1. The brain of rats from group 2 showed mild hydropic degeneration (Figure 3D). However, the brain tissues of animals from group 3 also showed mild hydropic deterioration similar to those found in animals that were administered with DF alone (Figure 3D). Light microscope analysis of the heart of group 1 rats showed standard histologic architecture with no specific changes (Figure 3E). However, rats treated with DF showed a minimal volume in the ventricle wall of the heart (Figure 3E). Rats from group 3 showed a slight increase in volume in the ventricle wall of the heart (Figure 3E) compared to group 2.

In the case of animals that were administered with a combination of DF and betaine, no significant histopathological alterations were observed, except a few lymphocytic infiltrates in the lungs, few sinusoids in livers, and very mild interstitial inflammation of renal tissue, mild hydropic degeneration in brain and decrease in the volume of ventricles in the heart. The results of the histopathological studies corroborated with that of the biochemical observations. The chemoprotective potential of betaine is evident from its ability to maintain the structural integrity of the liver and kidney of DF-treated rats. The hepatorenal protection of betaine may be attributed to its ability to enhance prostaglandin synthesis in the liver and kidney. Further, the toxicity of DF has also been attributed to its ability to induce oxidative stress. However, the present study did not assess whether betaine mitigates DF-induced oxidative stress. Thus, studies determining the transport and metabolism of betaine and its effects on DF-induced oxidative stress will aid in unveiling the intracellular mechanism of betaine.

CONCLUSION

In summary, our results indicated that betaine mitigates the adverse effects associated with DF-induced treatment in vivo in Wistar rats treated orally daily for 28 days. The protective effects of betaine may be attributed to its osmoprotective, anti-stress activity. It would be intriguing to carry out further studies to identify the mechanism underlying the anti-stress effects of betaine.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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