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European Journal of Experimental Biology, 2013, 3(2):371-378

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Effects of ascorbic acid on immunosuppressive drug-azathioprine-induced alteration in the liver and kidney of adult wistar rats [*Rattus norvegicus*]

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ABSTRACT

Azathioprine is a purine analogue immunosuppressive drug used to prevent organ rejection following organ transplantation and to treat a vast array of autoimmune diseases. In this work, we studied the effects of ascorbic acid on immunosuppressive drug-azathioprine-induced alterations in the liver and kidney of adult male Wistar rats. Thirty Rats with mean weight 210±2.65 g were randomly assigned into four-treatment and control groups [n=6]. The rats in the treated groups II and IV received 10 and 20 mg/kg/bwt of azathioprine, rats in the treated groups III and V received 10 and 20 of AZA in combination with 25 mg/kg/Bwt and 50 mg/kg/Bwt of ascorbic acid respectively, while rats in control group received normal saline orally for 21 days. After the expiration of the drug administration, rats were sacrificed; the liver and kidney were excised and fixed in Haematoxylin and Eosin for histological examinations. The tissue homogenate were assayed for activities of SOD and GPx, while the plasma was assayed for levels of ALT, AST, Urea and Creatinine. The histological findings of liver and kidney sections indicated that there were cyto-architectural distortions in the treated animals compared with the control animal. The treated groups show significant [p<0.05] decrease in homogenate SOD and GPx, however, plasma ALT, AST, Urea and Creatinine level significantly increased [p<0.05] in the treated animals compared with the control animals. Ascorbic acid could not ameliorate the untoward degenerative changes in the liver and kidney of azathioprine-induced alterations.

Keywords: azathioprine, ascorbic acid, liver, kidney, oxidative stress

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INTRODUCTION

The importance of immune system in protecting the body against harmful foreign molecules is well recognized. However, in some instances, this protection can result in serious problems. For example, the introduction of an allograft (that is, the graft of an organ or tissue from one individual to another who is not genetically identical) can elicit damaging immune response, causing rejection of transplanted tissue [1]. Transplantation of organ and tissue for example kidney, heart and bone marrow has become routine due to improved surgical techniques and better tissue typing.

Azathioprine is immunomodulatory drug often used to treat inflammatory bowel disease, autoimmune diseases, prevent rejection of transplanted organs and also used as anticancer drug. It is inhibitor of purine metabolism leading to DNA damage. Upon its administration, it is rapidly converted into several compounds, including the active 6-

mercaptopurine [2]. This positive effect does not necessarily come without a price, however. By suppressing the immune system, and lowering the number of infection-fighting white blood cells, Azathioprine makes individual more susceptible to infection while taking it. Azathioprine exists as either 25 mg or 50 mg oral tablet. Following oral ingestion of azathioprine, it is metabolized into the active 6-mercaptopurine, which itself is a purine synthesis inhibitor. 6-Mercaptopurine impedes DNA synthesis and thus inhibits the proliferation of cells, especially the fast-growing lymphocytes. T-cells and B-cells are particularly affected by the inhibition of purine synthesis [3]. Azathioprine is an effective drug used alone in certain autoimmune diseases, or in combination with other immunosuppressants in organ transplantation [3]. Azathioprine blocks the downstream effects of CD28 co stimulation. 6-MP [the active metabolite] interacts directly with GTP-binding protein Rac1, thus blocking up-regulation of BCL-xl mRNA and protein. Immediate or short-term side-effects are uncommon, but these may include nausea, fatigue, hair loss, and rash. Because azathioprine suppresses the bone marrow, patients will be more susceptible to infection. Acute pancreatitis can also occur, especially in patients with Crohn's disease [4].

Ascorbic acid or L-ascorbic acid is an essential nutrient for humans, in which it functions as vitamin. Ascorbate [an ion of ascorbic acid] is required for a range of essential metabolic reactions in all animals and plants. It is made internally by almost all organisms notable mammalian exceptions are most or all of the order chiroptera [bats], and the entire suborder Anthropoidea [5]. It is water soluble anti-oxidant which protects the body from oxidative stress, and also serves as a co-factor in several vital enzymatic reactions [6]. Thus, in this study, we investigated the morphologic and oxidative changes in the liver and kidney of adult male wistar rats exposed to immunosuppressive drug-azathioprine and possible protective effects of ascorbic acid.

MATERIALS AND METHODS

Drugs

A commercial available formulation of azathioprine [AZA] tablets 50 mg and ascorbic acid [Ascorbic acid] syrup were bought from Tuyil Pharmaceutical Industry, Ilorin, Kwara State. Each ml of ascorbic acid contains 20 mg of ascorbic acid.

Experimental Animals

The rats were purchased from Home-made Research Institute in Ilorin, Nigeria. They were housed within the Animal House of the Department of Anatomy, College of Health Sciences, University of Ilorin, in different cages at room temperature and maintained under a 12 h light/ 12 h dark cycle, with feeds and water available *ad libitum*. They were allowed to acclimatize for two weeks before the commencement of the experiment.

Experimental Design

A total of thirty [30] adult male Wistar rats of mean weight of 210±2.65 g were used for the experiment. At the commencement of experiment, the animals were assigned to five groups, each group containing six rats:

- Group I animals were given normal saline.
- Group II received AZA [10 mg/kg body weight] for 21 days.
- Group III received AZA [10 mg/kg body weight] + ascorbic acid [25 mg/kg body weight] for 21 days.
- Group IV received AZA [20 mg/kg body weight] for 21 days.
- Group V received AZA [20 mg/kg body weight] + ascorbic acid [50 mg/kg body weight] for 21 days.

The ascorbic acid, azathioprine and normal saline were administered as stated above orally, using a feeding tube [size 6], and the treatment lasted for 21 days.

Body weight

Body weights of the rats were taken prior to the administration of azathioprine and ascorbic acid and weekly thereafter.

Animal Sacrifice and Sample Collection

Twenty four hours after the 21st day of treatment, the animals were sacrificed by cervical dislocation. Blood samples were collected intracardially into lithium heparinized bottles for enzyme assay. The serum samples were used for biochemical analysis of AST and ALT activities using [7] method, also serum urea concentration was determined by the method of [8] while serum creatinine was determined using the Jaffe reaction [9].

A midline abdominal incision was made to open up the abdominal cavity and access the liver and kidney. The liver and right kidney were excised and weighed using an electronic sensitive analytical balance [Gallenkomp FA2104A,

England], then fixed quickly in formosaline and processed for light microscopic examination using haematoxylin and eosin stain [10].

The liver and left kidney were quickly weighed, homogenized in 0.25 M cold sucrose solution and centrifuged at 5000 rpm for five minutes. The supernatant which was stored at -80^{0}C used for the quantitative estimation of superoxide dismutase and glutathione peroxidase within 48hours. Trough spectrophotometry [Calorimetric method] the activity of superoxide dismutase [SOD] and glutathione peroxidase [GPx] were determined using the RANDOX's Kit [Antrium, UK]. GPx assay was carried out as described by [11] while SOD assay was done following the procedure described by [12].

HISTOPAHTOLOGY

After fixation, paraffin embedding was done, and 4 µm tissue sections were obtained using the Reichert-Jung 2050 rotary microtome, followed by hematoxylin and eosin for general cellular architecture [10]. The slides were then examined at magnifications of X1000 with 5.0 Mega Pixel eyepiece under a light microscope.

Statistical Analysis

Data were analysed using SPSS 16.0 [SPSS Inc, Chicago, USA] at $p<0.05$ and Excel 2007 [Microsoft Corporation, USA]. Data were expressed as mean±SEM. Means were compared using the students' t-test.

RESULTS

Weight Changes

It was observed that the administration of 10 mg/kg/bwt, 20 mg/kg/bwt of AZA and 25 mg/kg/bwt, 50 mg/kg/bwt of ascorbic acid and control preparation of normal saline on the testis of Wister rats resulted in a marked morphological changes.

There was relative gain in weight, weekly recorded for both the control and treated animals. The weight gain, however, was greatest in the control group and was statistically significant [$p<0.05$ df = 10] as shown in table 1.

Analysis of the average liver and kidney weight using t-test showed significant difference at [$P< 0.05$, df = 10] comparing the treated groups II, III, IV and V and the control group I. This is shown in the table 1 below.

TABLE 1: WEIGHTS CHANGES OF EXPERIMENTAL ANIMALS

Treatment groups	N	Initial weight [g]	Final weight [g]	Body weight difference [g]	Liver weight [g]	Kidney weight [g]
I	6	221±1.4*	330±10.8*	+109	11.44±2.7	1.07±0.3
II	6	184±1.5	219±1.5	+35	6.87±1.1	0.63±0.2
III	6	177±2.5*	237±2.5*	+60	5.32±0.4	0.42±0.0
IV	6	212±6.5	242±6.7	+30	9.76±0.3	0.64±0.0
V	6	195±1.4	226±1.4	+29	7.63±0.6	0.58±0.0

**Difference is statistically significant [p<0.05, n=6] compared with control group I*

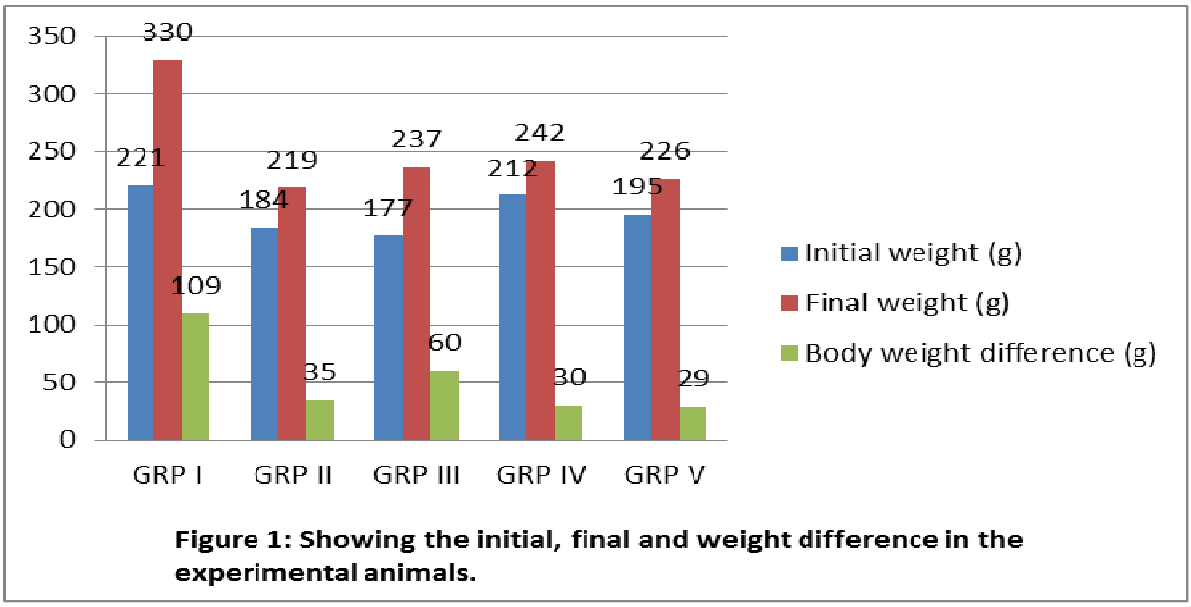

Figure 1: Showing the initial, final and weight difference in the experimental animals.

QUANTITATIVE ENZYME STUDIES

There were statistical increase in serum alanine aminotransferase [ALT], Aspartate aminotransferase [AST], urea and creatinine in animals exposed to azathioprine and ascorbic acid compared with the control animals. The increases were however, more in the 20 mg/kg/day of azathioprine and 50 mg/kg/day of ascorbic acid dose groups. The difference between treatments groups was statistically significant [p<0.05] as shown in figures 2, 3 & 4, and table 2.

Table 2: Results of ALT, AST, Urea, Creatinine, SOD & GPx activities.

	Treatment groups				
	I	**II**	**III**	**IV**	**V**
ALT [IU/L]	38.58 ± 74	45.09 ± 84*	41.01 ± 32	60.86 ± 04*	59.06 ± 33*
AST [IU/L]	64.07 ± 14	69.33 ± 29	61.91 ± 02	81.97 ± 72*	74.02 ± 29*
Urea [mmol/L]	3.2 ± 0.2	5.1 ± 0.1	4.4 ± 0.1	6.3 ± 0.5*	5.7 ± 0.4*
Creatinine [mmol/L]	43.5 ± 3.5	74.5 ± 5.5*	52.0 ± 1.0	61.5 ± 1.5*	62.0 ± 3.0*
SOD [U/g protein]	36.54 ± 2.8	23.86 ± 7.0*	32.54 ± 8.9	12.32 ± 6.5*	17.98 ± 0.5*
GPx [mU/ml protein]	85.54 ± 12	67.15 ± 30*	73.39 ± 76	51.05 ± 16*	58.20 ± 87*

*Data are represented as Mean ±S.E.M [n=6]. Significance level at *[P≤ 0.05].*

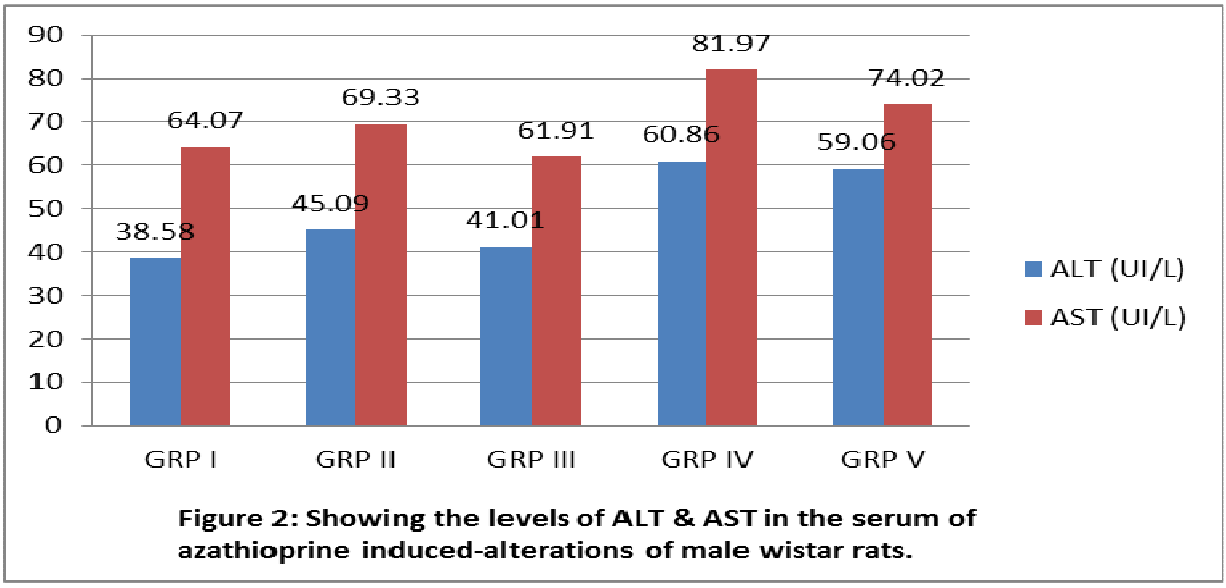

Figure 2: Showing the levels of ALT & AST in the serum of azathioprine induced-alterations of male wistar rats.

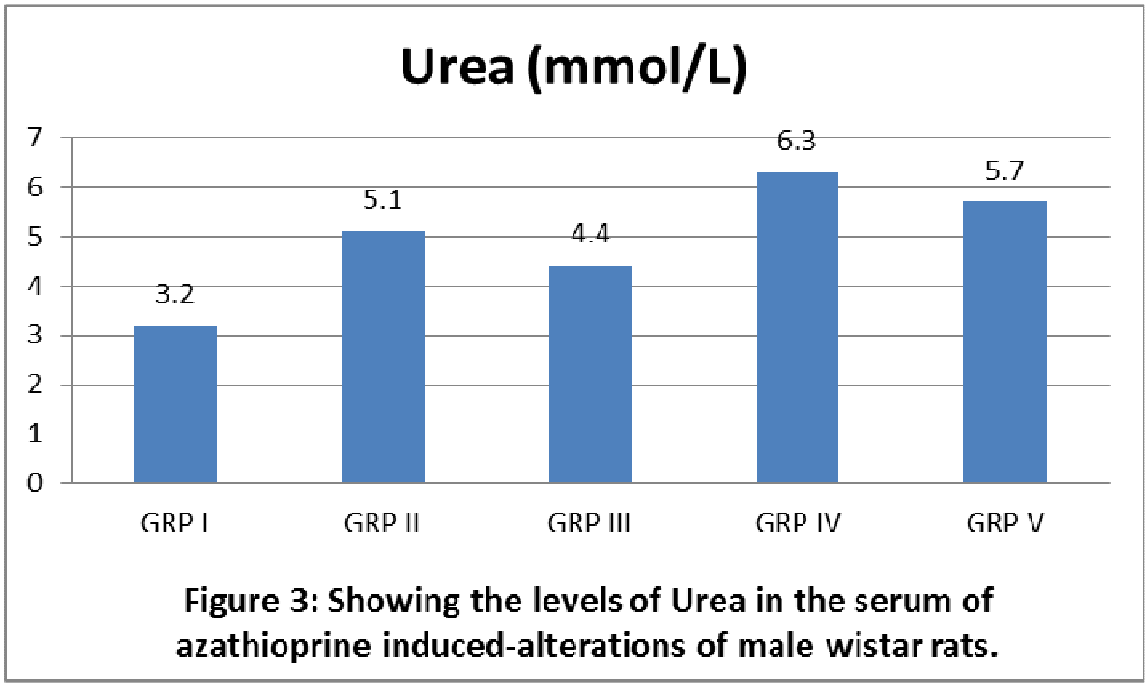

Figure 3: Showing the levels of Urea in the serum of azathioprine induced-alterations of male wistar rats.

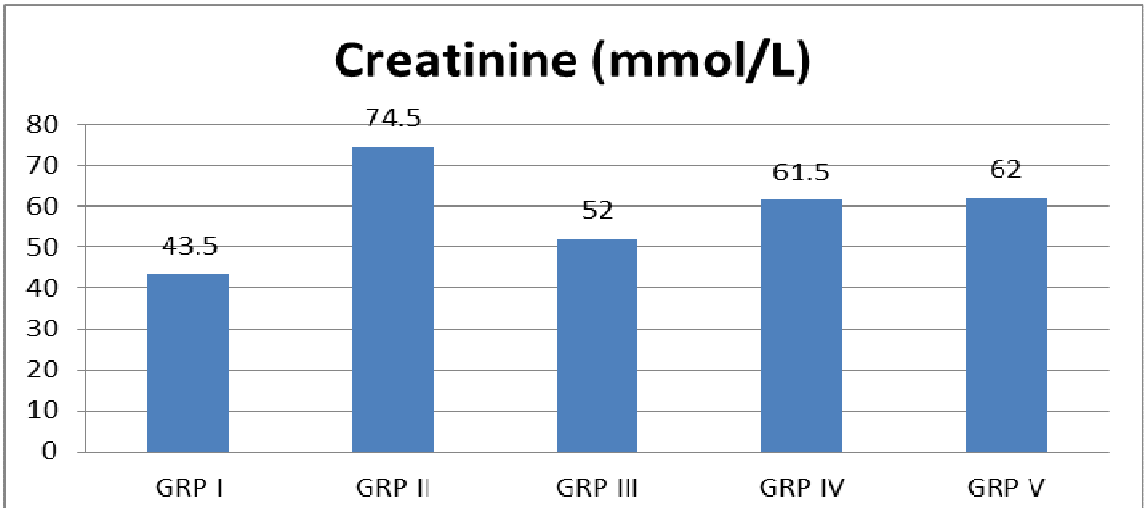

Figure 4: Showing the levels of Creatinine in the serum of azathioprine induced-alterations of male wistar rats.

The level of homogenate SOD and GPx were decreased in animals administered with azathioprine only and combined azathioprine with ascorbic acid compared with control group. These were however statistically significant at $p<0.05$ as shown in figure 5 and table 2.

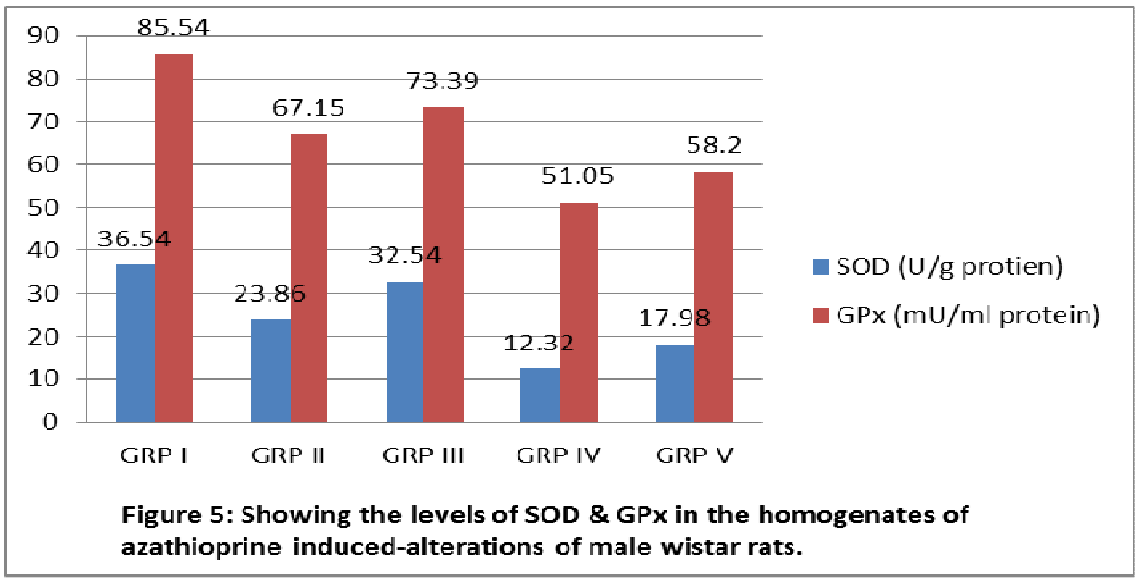

Figure 5: Showing the levels of SOD & GPx in the homogenates of azathioprine induced-alterations of male wistar rats.

HISTOLOGICAL OBSERVATIONS:

Fig. 6 shows the histologic findings of haematoxylin and eosin stained liver sections. In the control group, no necrotic changes or inflammatory cells were observed [Fig. 7, I]. In the experimental groups, hepatocytes necrosis concomitant with inflammatory cells infiltration were observed in different portions of the hepatic lobules. In the treated group II [10 mg/kg of AZA] and group III [10 mg/kg of AZA+25 mg/kg of ascorbic acid] with hepatocytes necrosis was prominent but the infiltration by inflammatory cells was reduced when compared to the group IV [20 mg/kg of AZA] and group V [20 mg/kg of AZA+50 mg/kg of ascorbic acid] [Fig.s 7, II-V].

Histological findings in the control kidney reveal an intact glomeruli, proximal convoluted tubule and distal convoluted tubule [Fig. 7, I]. Similarly in the experimental groups, an intact renal corpuscle, proximal convoluted tubule [PCT] with brush borders, undisrupted distal convoluted tubule is also noticed with clearly seen macula densa [Figs. 7, II-V]. Collagen type IV of the basement membrane of the glomerular capillaries is clearly visible in all the groups.

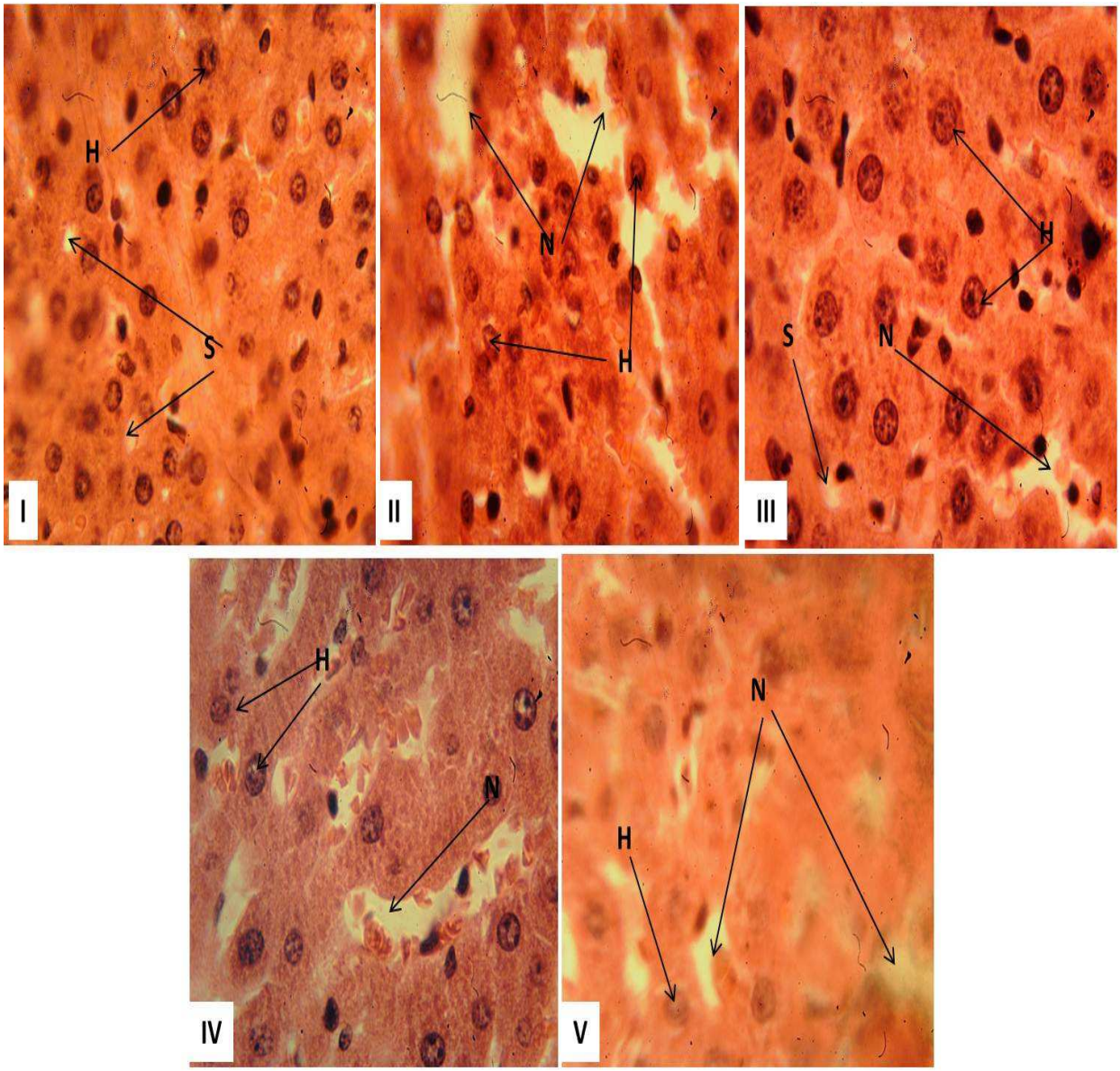

Figure 6 (I-V): Hepatic histology after 28 days treatment. I: Control; II: 10 mg/kg of AZA, III: 10 mg/kg AZA+25 mg/kg of Ascorbic acid, IV: 20 mg/kg of AZA, V: 20 mg/kg of AZA+50 mg/kg of Ascorbic acid. **KEYS: H = Hepatocytes; N = Areas showing necrosis of hepatocytes with inflammation; S = sinusoid. (H&E ×1000).**

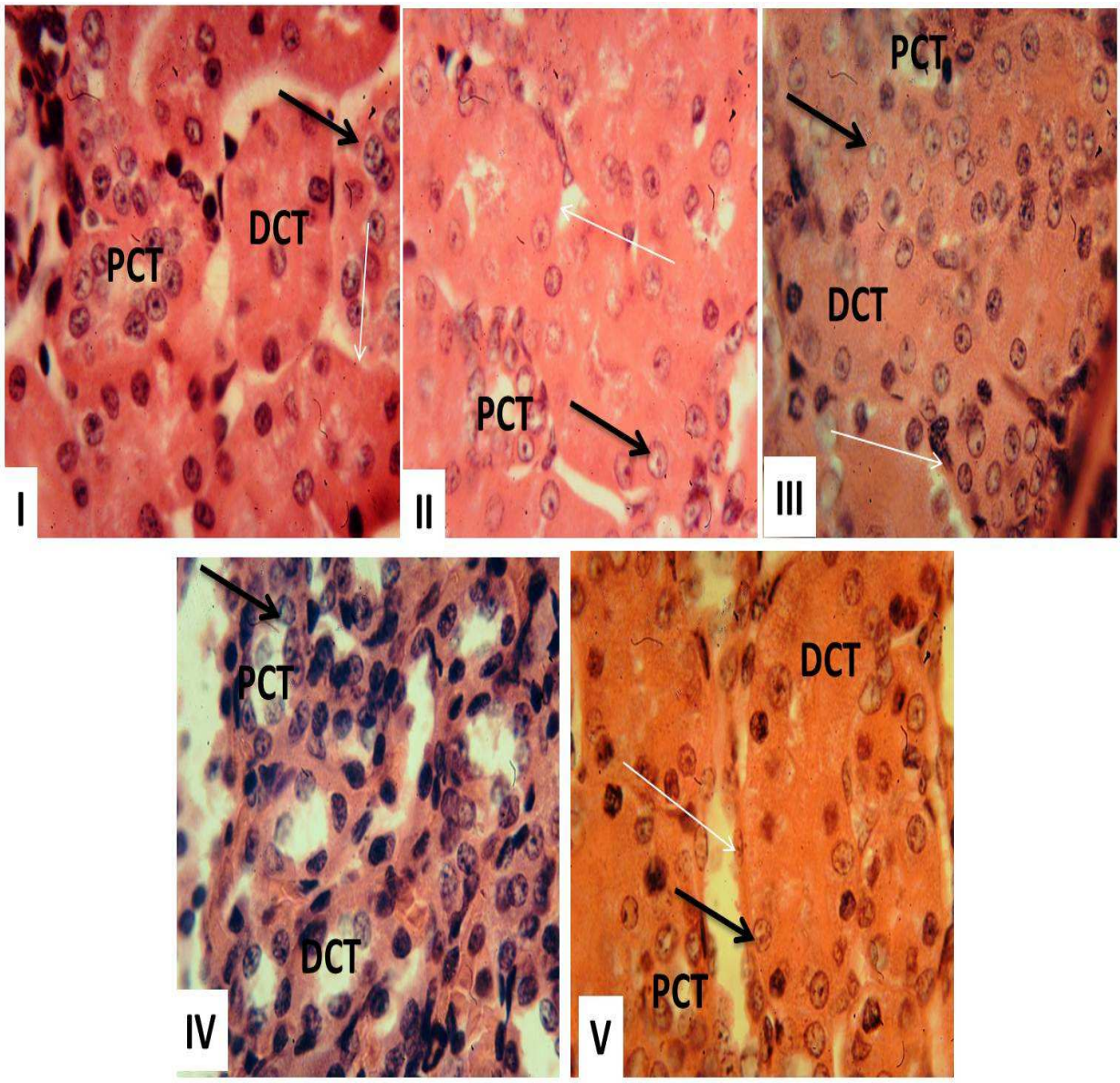

Figure 7 (I-V): Photomicrograph of the renal cortex of experimental animals showing intact proximal convoluted tubule (PCT) with its large cuboidal cells presenting a brush border. The distal convoluted tubule (DCT) is also intact. A macula densa is clearly seen (black arrow). The collagen type IV of the basement membrane of the glomerular capillaries is clearly visible (white arrow) (H&E X1000).

DISCUSSION

In this study, results from biochemical and histopathological assessments showed hepatic injuries and kidney damage in rats with azathioprine-induced alterations. Significant increase [P≤0.05] in serum levels of ALT, AST, urea and creatinine were observed in comparison with normal control rats [Table 2]. The oral administration of the ascorbic acid for four consecutive weeks could not reverse these liver and kidney function markers to normalcy. Serum levels of ALT and AST are widely used to evaluate liver function. Increased levels of these enzymes are indicators of cellular infiltration and functional disturbances of liver cell membranes [13]. The persistent increase in the activities of these enzymes in the serum following administration of ascorbic acid may not be attributed to the prevention of these intracellular enzymes leak from either cell membrane stability or cellular regeneration.

There were significant increase [P≤0.05] in the serum levels of urea and creatinine in the extract treated groups compared with the control as shown in Tables 2. Urea and creatinine are waste products which are passed into the blood stream to be removed by the kidney. Elevation of these waste products in the blood is an indication of renal function impairment [14, 15]. Serum levels of these metabolites in the test groups were significantly increased when

compared with the control which showed that the ascorbic acid did ameliorate derangement caused by azathioprine in the cellular activities of the rat's kidneys.

SOD and GPx are antioxidant enzymes which establish the defensive system against reactive oxygen species [ROS] [16]. Reduction in SOD activity is an important index for injuries caused by ROS. SOD eliminates superoxide anions by converting them to hydrogen peroxide [H_2O_2] hence reducing their toxic effects [17]. Glutathione peroxidase [GPx] is a selenium containing enzyme present in significant concentrations that helps detoxifies H_2O_2 to H_2O through the oxidation of reduced glutathione [18]. In the present study, both SOD and GPx activities are significantly reduced in the azathioprine-induced alterations groups when compared to the control group due to the continual production of superoxide anions. In accordance Zeinab [19] who reported that this reduced antioxidant production may be due to increase in oxygen metabolite that causes a decrease in the activity of the antioxidant defense system. In this study the oral administration of the ascorbic acid did not elevated the activities of SOD and GPx when compared with the control group. Though, ascorbic acid was reported to be water soluble anti-oxidant which protects the body from oxidative stress, and also serves as a co-factor in several vital enzymatic reactions [6] but this could not be achieved in the cause of this study, and this may be due to unknown mechanism of severity of damage resulting from administration of azathioprine.

With the oral administration of ascorbic acid at the dose of 25 mg/kg and 50 mg/kg, only mild infiltrative activities of inflammatory cells were observed histologically in the liver and there was necrotic changes indicating that ascorbic acid is not potent in ameliorating the damages resulting administration of azathioprine. However, pathologic findings are matched with biochemical results. Histologically, the renal corpuscles, proximal convoluted tubule and distal convoluted tubules of the extract treated groups show no distortion in their cyto-architecture when compared with the control group.

CONCLUSION

Our study suggests that ascorbic acid administered to rats did not ameliorate cellular disruption to the histology of the liver and kidney function indicators resulting from administration of azathioprine. We therefore conclude that the administration of azathioprine is deleterious on the liver and kidney of rats which could imply its possible side effects in it use for prevention of organ rejection and autoimmune diseases in humans.

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