

Effect of 1,5-Bis (3,5-dimethylpyrazol-1-yl)-3-oxapentanediacetatocopper on kidney and spleen of rats: Histological and immunohistochemical evaluation

Abstract

The present work was carried out to evaluate the histological and immunohistochemical changes of the kidney and spleen of rats after repeated exposure to 1,5-Bis (3,5-dimethylpyrazol-1-yl)-3-oxapentanediacetatocopper (BDO). Two groups of male albino rats were used. The first group is the control and the second group was i.p. given 1,5-Bis(3,5-dimethylpyrazol-1-yl)-3-oxapentane diacetatocopper (12 mg / kg body weight) daily for 6 weeks. The results showed that the administration of BDO to rats exhibits various histological effects on tissues of kidney and spleen. Kidney from treated animals showed congestion of blood vessels, degeneration of renal tubules and glomeruli. The spleen tissue lost its characteristic structure and showing interference \ overlap between red and white pulp, enlarged trabecula and degenerated lymphocytes. Histochemical results revealed depletion of total proteins contents in renal tubules and glomeruli and spleen cells of treated rats. Expression of Bcl-2 protein was increased in the kidney and spleen of treated rats. It is concluded from the present work that BDO induced histopathological alterations and apoptosis in kidney and spleen of rats.

Keywords: Dimethylpyrazol, Histology, Bcl-2 protein, kidney, spleen, Rats.

Introduction

The search for new anti-diabetic drugs is one of the most important problems of modern medicine. A lot of pyrazole derivatives exhibit various pharmacological properties and are characterized by an antioxidant, antiviral activity, anti-inflammatory, immunomodulating properties, as well as antidepressive and anti-tumor effect (Choia *et al.*, 2013). It is also known that complexes of copper (II) with pyrazole derivatives exhibit biological activity as regulators of reactive oxygen species and superoxide dismutase (Potapov *et al.*, 2009). The action 3,5-dimethylpyrazole is similar to that of insulin in intact rats (Cavallini *et al.*, 2014).

1,5-Bis(3,5-dimethylpyrazol-1-yl)-3-oxapentanediacetatocopper ($C_{18}H_{28}N_4O_5-Cu$) is one of the new derivatives and the most representative of pyrazole-containing copper (II) complexes, which was synthesized in the Polzunov Altai State Technical University (AltSTU) in 2007 year (Potapov *et al.*, 2007). 1,5-Bis(3,5-dimethylpyrazol-1-yl)-3-oxapentanediacetatocopper is a hypoglycemic agent which decreased the level of glucose in the blood and are used in the treatment of diabetes mellitus (Nofal *et al.*, 2015).

The administration of antilipolytic drug (3,5-dimethyl-pyrazole at dose 12 mg/kg body weight) to rats affected both lipid and glucose metabolism of the animal in a very short time, this drug caused a dramatic and prolonged decrease in the plasma levels of free fatty acids and significantly lower glucose plasma levels (Bergamini *et al.*, 1987). The present work studied the effect of 1.5-Bis (3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper on kidney and spleen of rats.

Materials and methods

1. 1.5-Bis (3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper

1.5-Bis(3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper was dissolved in 0.9% physiological saline solution and injected intraperitoneally at dose 12 mg/kg body weight /daily for 6 weeks (Cavallini et al., 2014).

2. Animals and treatments

In this study, adult male albino rats (*Rattus norvegicus*) weighing $130 \pm 5g$ were used. Rats were breed in the Animal House of the Department of Zoology / Faculty of Science, Menoufia University) and placed under constant temperature ($24 \pm 2^\circ C$) throughout the experimental work. They were maintained on a standard rodent diet composed of 20% casein, 15% corn oil, 55% corn starch, 5 % salt mixture and 5% vitaminized starch. Water was available *ad libitum*. Maintenance of animals and experimental procedures was approved by the animal ethical committee in accordance with the guide for care and use of laboratory animals. Animals were divided into 2 groups:

First group; animals of this group served as a control group.

Second group: animals of this group were injected intraperitoneally 1.5-Bis(3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper dissolved in saline solution at a dose level of 12 mg/kg body weight /daily for 6 weeks.

3. Histological study

For histological study, animals were sacrificed after 6 weeks; kidney and spleen were immediately removed and fixed in 10% neutral formalin for

24 hours. After fixation, specimens were dehydrated in ascending series of ethyl alcohol, cleared into two changes of xylene, infiltrated in three changes of molten paraffin wax with melting point of 58- 60°C and then embedded in molten paraffin blocks. Sections of 5 microns thickness were cut by using rotary microtome and mounted on clean slides. For histological examination, sections stained with Ehrlich's hematoxylin and counter stained with Eosin (Lillie and Fulmer, 1976).

4. Histochemical procedures

For histochemical purposes, small pieces of kidney and spleen were fixed in 10% neutral formalin for demonstration of total protein. Sections of 5 microns thickness were cut. Total proteins demonstrated by mercury bromophenol blue method (Pearse, 1972).

5. Immunohistochemical study

The Bcl-2 protein was detected by the immunoenzymatic alkaline phosphatase anti-alkaline phosphatase method, using an anti-human Bcl-2 monoclonal antibody (DAKO A/S, Glostrup, Denmark) (Cordell et al., 1984). The sections were deparaffinized and rehydrated routinely. Antigens were retrieved by heating the sections in a microwave oven at 700 W in 10 mmol/L citrate buffer (pH 6.0) for 10 min. After blocked with 3 mL/L H₂O₂ and swine serum, specimens were then incubated with the primary antibodies, directed against Bcl-2. The staining was performed by streptavidin peroxidase enzyme conjugate method using a S-P kit (Zymed). Reaction products were visualized by DAB. Brown-yellow granules in cytoplasm were recognized as positive staining (Cordell et al., 1984).

Image analysis

Digital images were analyzed by a semi-quantitative scoring system (Image J software, Java based application for analyzing images). The immune-stained sections were analyzed in 10 microscopic fields under high-power field (×400) microscope. In each field, the immunopositive (brown) area was recorded. Percentage of positive stained area (%) was calculated as mean of 10 fields / slide.

6. Statistical analysis

Data were expressed as mean ± standard error (SE). The significance of differences means evaluated by using independent sample t-test. Statistical

program of social sciences (SPSS) software for windows, version 20 was used.

Results

1- Histological observation

a. Kidney

Examination of the kidney sections of control rat revealed normal structures of the renal cortex which consists of renal corpuscles, proximal and distal convoluted tubules. Renal corpuscles consist of Bowman's capsule is enclosed with a tuft of glomerular capillaries and double membrane with urinary space in between (Fig.1a).

Kidney sections of rats treated with BDO for 2 weeks showed degeneration and deterioration of the cortical constituents with damaged tubule, as well as, enlarged and congested renal veins (Fig.1b). The Malpighian corpuscles after 4 weeks of treatment lost their characteristic configuration with atrophied and fragmented glomerulus, moreover, the epithelial cells lining renal tubules were exfoliated from their underlying basement membrane and degenerated with vacuolated cytoplasm and pyknotic nuclei, as well as, enlarged and congested renal veins was observed (Fig.1c). While, after 6 weeks, a number of glomerular capillaries were suffering from severe signs of glomerular congestion, atrophy, fragmentation and degeneration, while others were lost their attachments and mesangial stroma. Congestion and dilatation of blood vessel were observed, moreover, the renal tubules suffering from degeneration and their lumen were filled with proteinaceous casts (Fig.1d).

b. Spleen

The normal spleen in the control group displayed splenic parenchyma composed of white and red pulp surrounded by a capsule of dense connective tissue, from which emanated trabeculae dividing the splenic parenchyma into incomplete compartments (Fig.2 a). The spleen sections of animals treated with BDO for 2 weeks showed low lymphocyte densities, more fibroblasts, more trabeculae and the lymphocytes appeared with pyknotic nuclei (Fig.2 b). After 4 weeks of treatment, the spleen tissue lost its characteristic structure and showing interference \ overlap between red and white pulp, enlarged trabecula (Fig.2 c). These changes were more apparent in spleen sections examined after 6 weeks. In these specimens, congestion of splenic cords and sinuses and dilated congested blood vessel were observed. The trabeculae were enlarged and most of the lymphocytes were damaged with pyknotic nuclei (Fig.2 d).

2- Histochemical results

In control kidney of rat, the protein materials are positively stained in the form of small bluish irregular particles in cytoplasm of the cells of renal tubules, the nuclear envelope, chromatin materials and nucleoli (Fig. 3a). Examination of kidney of rats after 2, 4, 6 weeks of treatment with BDO showed a gradual reduction of protein content of the cells lining tubular epithelia and the glomeruli (Figs. 3 b,c,d).

The protein materials of spleen of control rat are positively stained in the form of small bluish irregular particles in cytoplasm of all the cells, the nuclear envelope, chromatin materials and nucleoli (Fig. 4a). Examination of spleen of rats after 2, 4, 6 weeks of treatment with BDO showed a reduction of protein content of all cells (Figs. 4 b,c,d).

3- Immunohistochemical results

Normal kidney rat demonstrated Bcl-2 immunopositivity primarily within the cytoplasm cells of distal tubule and rarely staining of the proximal tubular (Fig. 5a). In contrast, Bcl-2 protein expression was increased in cytoplasm cells of renal tubules of kidney rats after 2, 4, 6 weeks of treatment with BDO (Figs. 5 b,c,d).

Spleen of normal rat demonstrated rarely Bcl-2 immunopositivity staining (Fig. 6a). In contrast, Bcl-2 protein expression was increased in spleen rats after 2, 4, 6 weeks of treatment with BDO (Figs. 6 b,c,d).

Image Analysis of immunohistochemical reaction of Bcl-2

Treating rats with BDO for 6 weeks resulted in a significant increase ($p < 0.01$) in the Bcl-2 protein expression in spleen and kidney when compared with the normal control group (Table, 1).

Table (1): The mean percentage area of Bcl-2 positive staining in kidney and spleen of rats in control and BDO groups.

Animal groups	Spleen	Kidney
Control	0.592± 0.008	2.712± 0.005
2 weeks	2.721± 0.005*	12.998± 0.009*
4 weeks	5.822± 0.008**	20.118± 0.01**
6 weeks	32.518± 0.01**	37.051± 0.004**

Data presented as means ± standard errors

(*) Significant difference as compared with control group at $P < 0.05$

(**) Significant difference as compared with control group at $P < 0.01$

Discussion

The results of the present investigation demonstrated the adverse effect of 1,5-Bis(3,5-Dimethylpyrazol-1-yl)-3-oxapentanediacetatecopper on kidney and spleen of rat. Exposing rats to 1,5-Bis(3,5-Dimethylpyrazol-1-yl)-3-oxapentanediacetatecopper for 6 weeks was found to induce different histopathological alterations. The kidney showed congestion of renal blood vessels, degeneration of renal tubules and glomeruli. The spleen appeared with congestion of splenic blood vessels, low lymphocyte densities, more fibroblasts, more trabeculae and degeneration of cellular components. Similarly, Nofal *et al.* (2014) reported that liver sections after treatment with the same drug (BDO) manifested loss of normal hepatic structure, inflammatory infiltration, marked enlarged vacuolated cytoplasm in hepatic cells, congestion of blood vessels.

These alterations seemed to follow almost the same pattern as that previously enumerated by some investigators under the effect of different derivatives of pyrazole. Wilson and Bottiglieri (1962) reported that pyrazole induced primary toxicity in the liver, kidney and bone marrow of human. Intraperitoneal injection of pyrazole enhanced liver injury, oxidative stress, induction of apoptosis and necrosis (Lu and Cederbaum, 2006). Pyrazole induced oxidative liver injury in mice (Bae *et al.* 2012). The administration of 3,5-dimethylpyrazole at dose 12 mg/kg body weight revealed many vacuolated lysosomes and autophagic vacuoles in the liver cells of rats (Bergamini *et al.*, 1987).

Histochemical results revealed depletion of total proteins contents in kidney and spleen of rats treated with 1,5-Bis(3,5-Dimethylpyrazol-1-yl)-3-oxapentanediacetatecopper for 6 weeks. Also, the administration of 3,5-dimethylpyrazole at dose 12 mg/kg body weight caused intracellular degradation of proteins in rat liver cells (Bergamini *et al.*, 1987). The administration of rat with 3,5dimethylpyrazole at dose 12mg/Kg body weight decreased significantly the peroxisomal enzyme activities and increased degradation of liver proteins (Bergamini and Segal, 1987). Lindros *et al.* (1983) reported that chronic treatment rats with the higher dose of 4-methylpyrazole caused a relative decrease in the liver protein content.

Apoptosis is an essential physiological process for the selective elimination of cells, which is involved in a variety of biological events. The Bcl-2 family is the protein family involved in the regulation of apoptotic cell death. Bcl2 is anti-apoptotic members prevent apoptosis either by sequestering proforms of death-driving cysteine proteases called caspases (a complex called the apoptosome) or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and AIF (apoptosis-inducing factor)

into the cytoplasm. After entering the cytoplasm, cytochrome c and AIF directly activate caspases that cleave a set of cellular proteins to cause apoptotic changes (Tsujimoto, 1998). In the present work, treating rats with 1,5-Bis(3,5-Dimethylpyrazol-1-yl)-3-oxapentanediacetatecopper for 6 weeks caused marked expression of Bcl-2 in the kidney and spleen cells. In agreement with this result, Nofal *et al.* (2014) revealed increase expression of Bcl-2 proteins in hepatic cells of rats treated with the BDO. Bcl-2 protein was exclusively positive expressed by ductular epithelial cells (Frederic *et al.*, 1994). Bcl-2 protein is overexpressed in the majority of renal cell carcinomas and plays a role in tumorigenesis (Andrew *et al.*, 1999). It is concluded from the present work that BDO induced histopathological alterations and apoptosis in kidney and spleen of rats.

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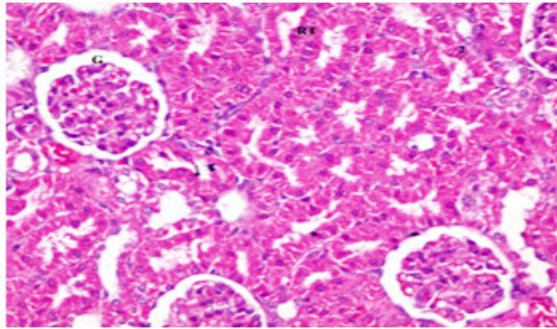
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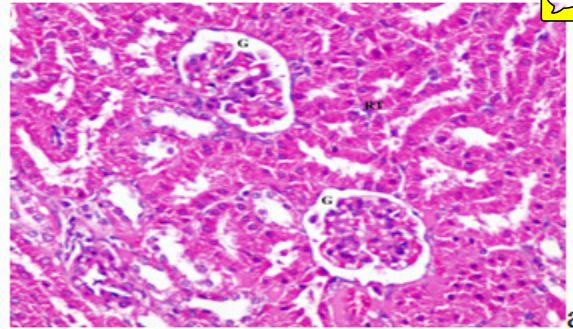
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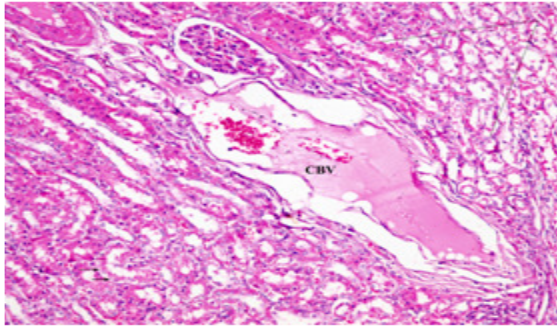
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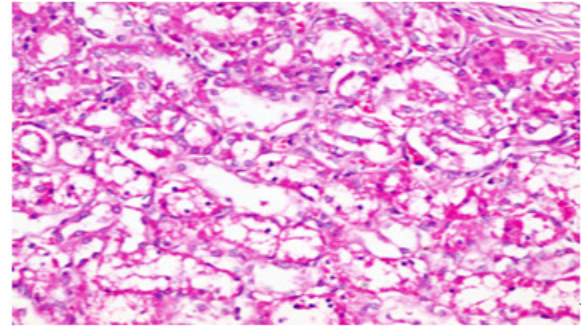
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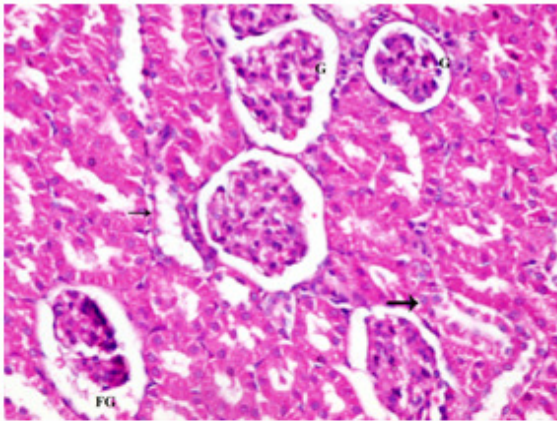
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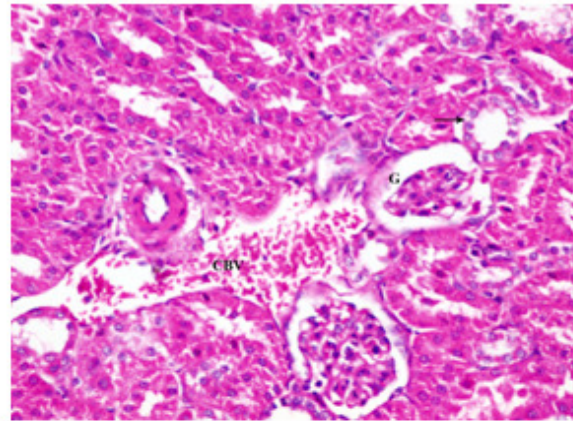
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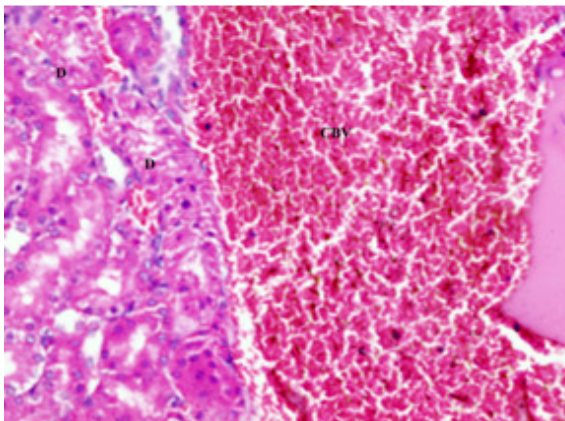
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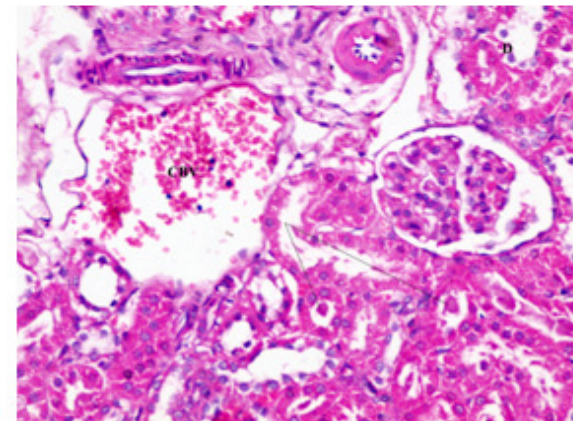
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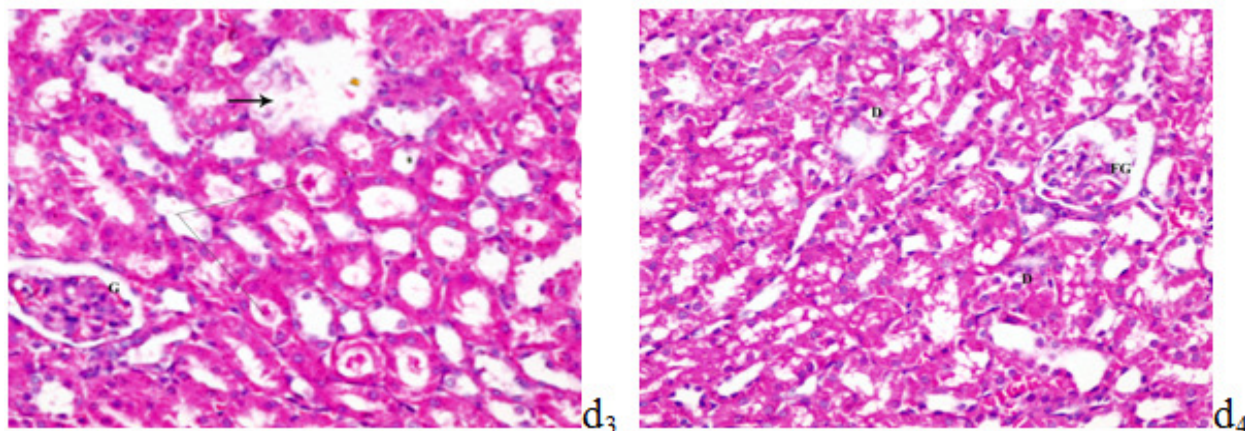


Figure 1(a): Kidney of a control rat showing the glomerulus (G) and renal tubules (RT), X 400.

Figure 1(b): Kidney of rat after treatment with 1.5-Bis(3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper for 2 weeks showing congested blood vessel (CBV), damaged tubule (double arrows), X 400.

Figure 1(c): Kidney of rat after 4 weeks showing exfoliation of epithelial cells of some renal tubules with vacuolated cytoplasm and pyknotic nuclei (arrows), atrophied glomerulus (G), Fragmented glomerulus (FG), congested blood vessel (CBV), X 400.

Figure 1(d): Kidney of rat after 6 weeks showing congested blood vessel (CBV), damaged tubule (D), atrophied degenerated glomeruli (G), proteinaceous casts in the lumen of the renal tubules (double arrows), edema (arrow), Fragmented glomerulus (FG), X 400.

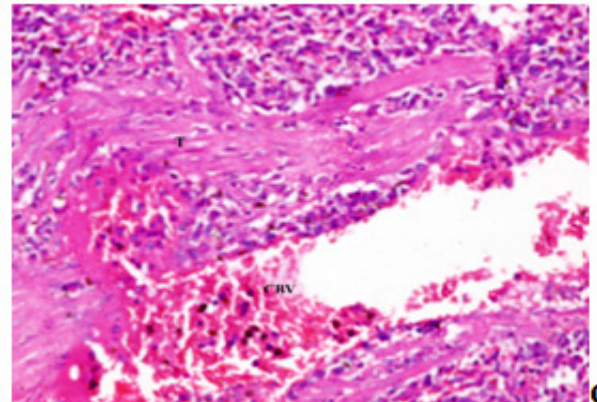
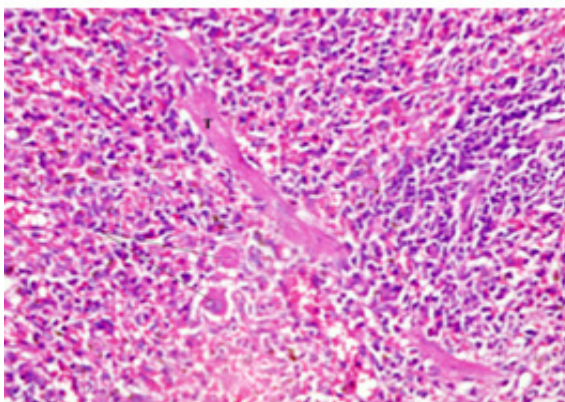
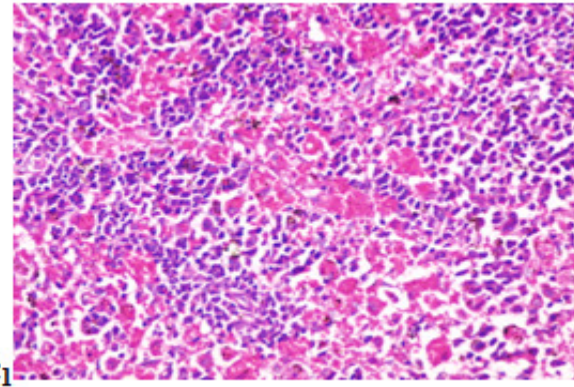
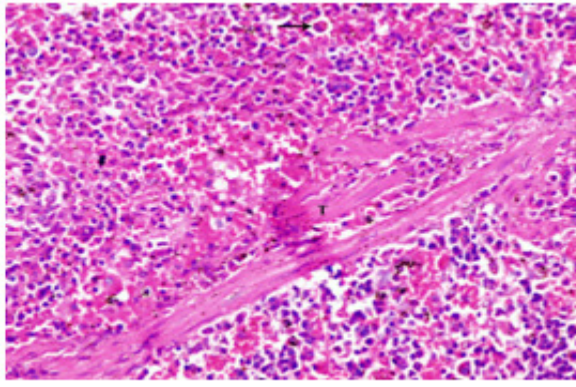
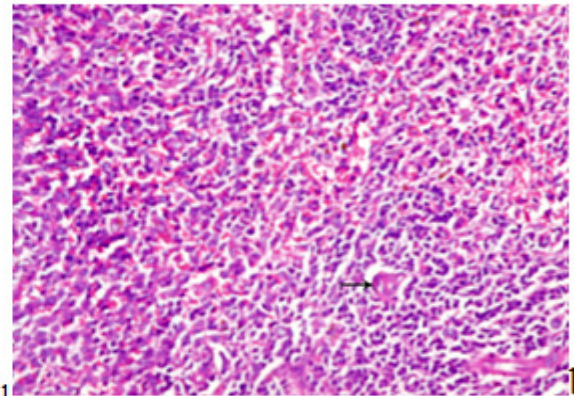
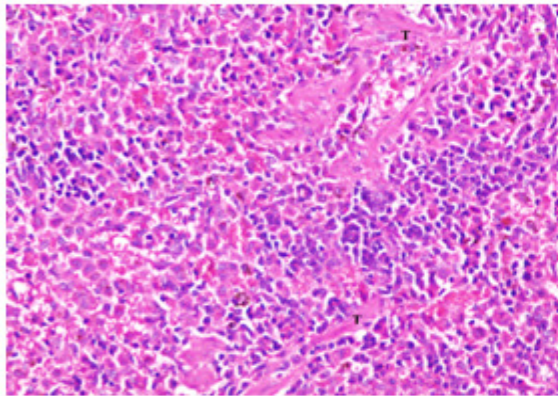
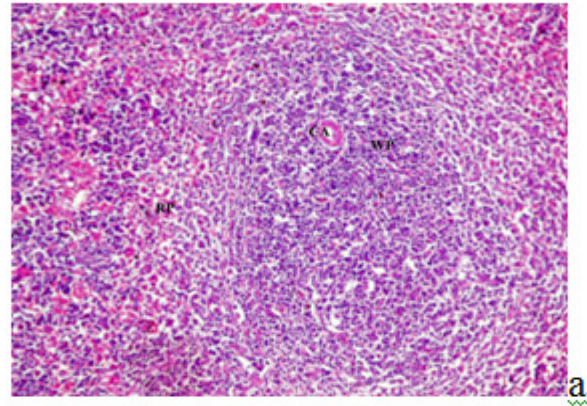


Figure 2(a): Spleen of a control rat showing red pulp (RP), white pulp (WP) and central arteriole (CA), X 400.

Figure 2(b): Spleen of rat after treatment with 1.5-Bis(3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper for 2 weeks showing fibroblasts, large bundles of fibers of trabecula (T) and pyknotic nuclei (arrow), X 400.

Figure 2(c): Spleen of rat after 4 weeks showing interference \ overlap between red and white pulp, enlarged trabecula (T), X 400.

Figure 2(d): Spleen of rat after 6 weeks showing dilated congested blood vessel (CBV), enlarged trabecula (T), congestion of splenic cords and sinuses and pyknotic nuclei (double arrows), X 400.

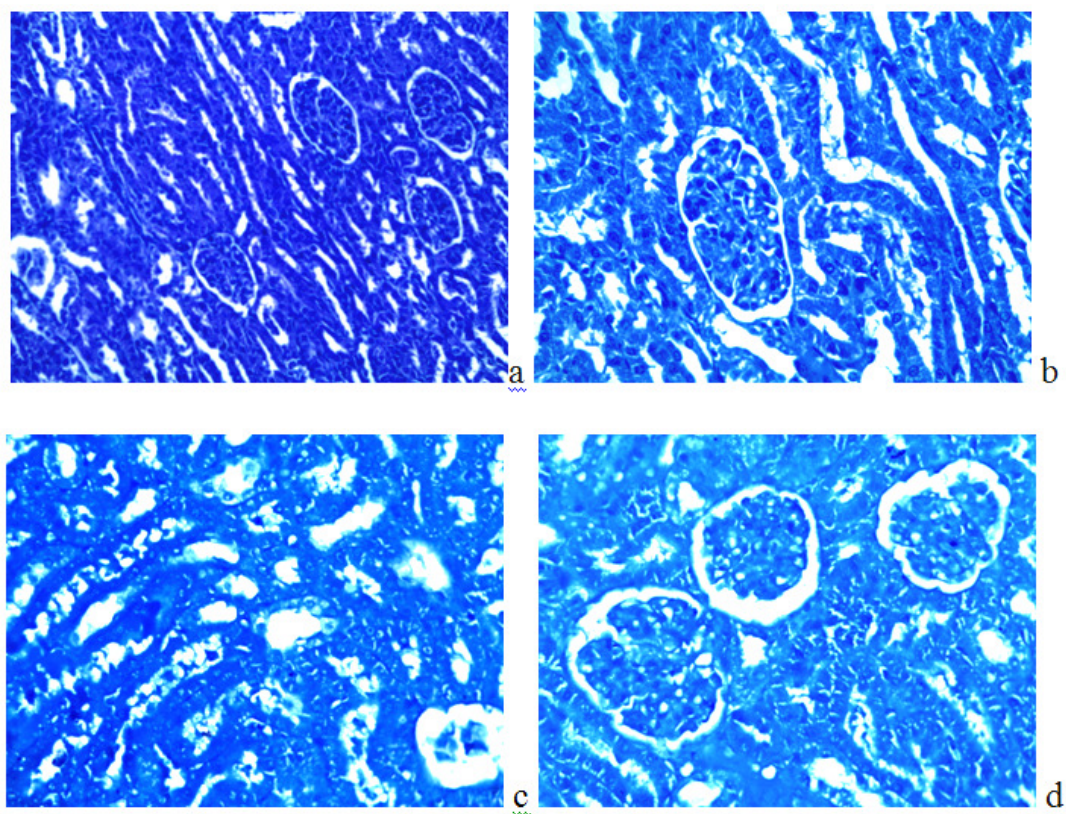


Figure 3(a): Normal protein content in renal tubules and glomeruli of a control rat, X 400.

Figure 3(b,c,d): Marked reduction of total proteins in kidney rats treated with 1.5-Bis(3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper, X 400.

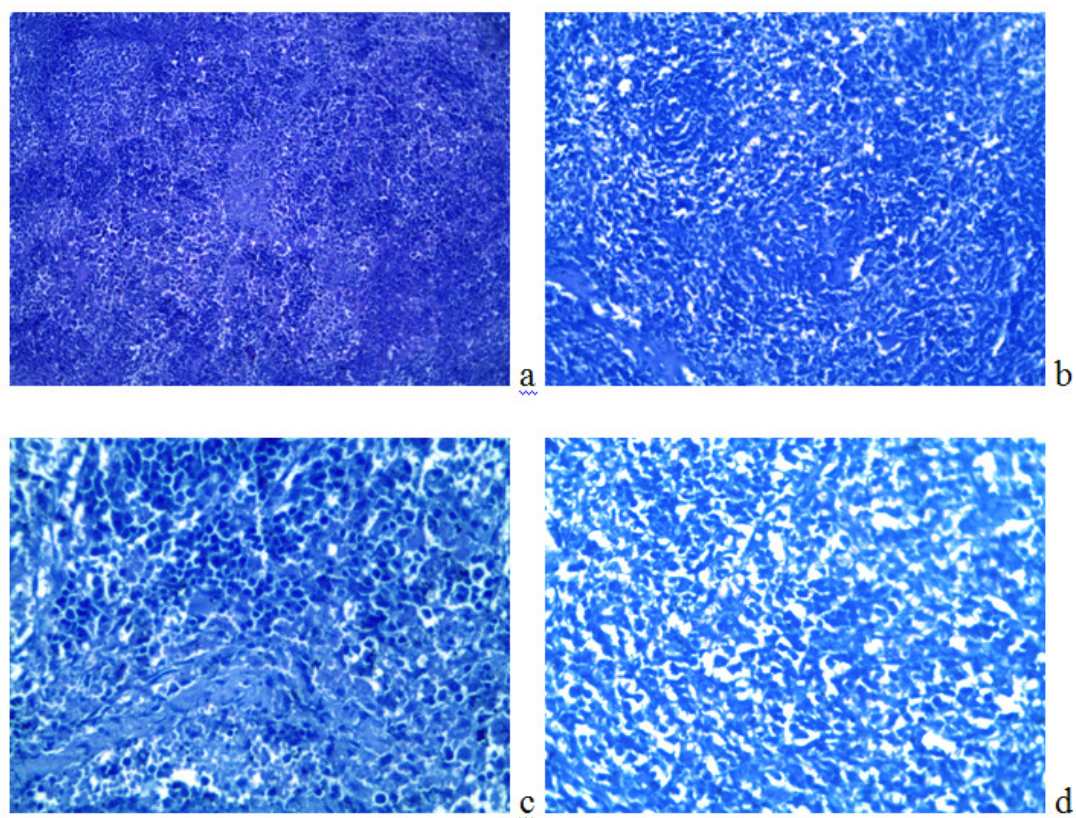


Figure 4(a): Normal protein content in spleen of a control rat, X 400.

Figure 4(b,c,d): Marked reduction of total proteins in spleen rats treated with 1.5-Bis(3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper, X 400.

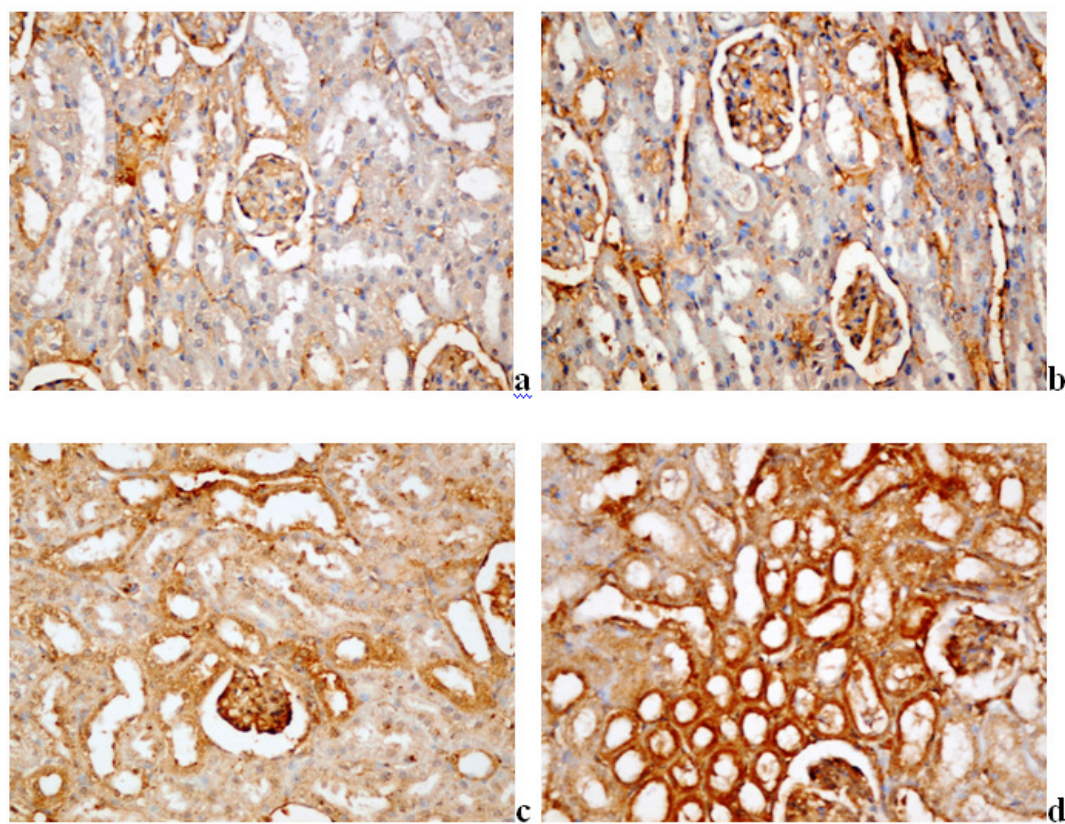


Figure 5(a): Kidney section of a control rat showing few expression of Bcl-2 (Immunostain, X 400).

Figure 5(b,c,d): Increase in expression of Bcl-2 in kidney of a rat treated with 1.5-Bis(3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper (Immunostain, X 400).

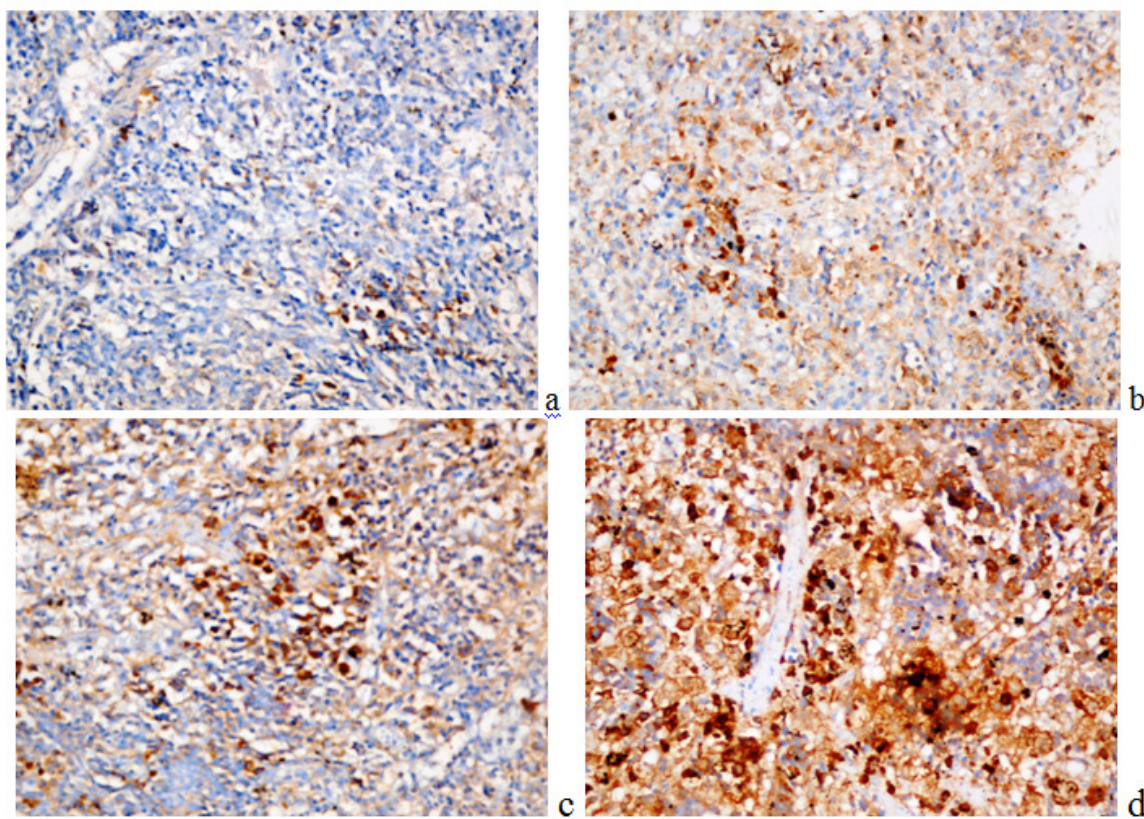


Figure 6(a): Spleen section of a control rat showing few expression of Bcl-2 (Immunostain, X 400).

Figure 6(b,c,d): Increase in expression of Bcl-2 in spleen of a rat treated with 1.5-Bis(3.5-Dimethylpyrazol-1-yl)-3-oxapentane-diacetatocopper (Immunostain, X 400).