

Protective Effects of Ascorbic Acid and Garlic Oil against Toxic Effects induced by Sodium Nitrite as Meat Additive in Male Rats

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Abstract: Sodium nitrite has been widely used for decades as a meat additive for preservation of meat products. The present study investigated the protective role of ascorbic acid and garlic oil 2% against toxic hematological, serological and histopathological effects of sodium nitrite used as meat additive in manufacturing of kofta fed to male albino rats. Manually processed kofta supplemented with different concentrations of nitrite and ascorbic acid was fed to male rats for 30 days. Control group received manually processed kofta without any additives. There was significant decrease in RBCs count, HG content and some hematological parameters revealing anemia, as well as significant increase in AST, ALT, urea and creatinine levels was detected due to nitrite consumption. On the other hand, ascorbic acid and garlic oil ameliorated to a high extent, the anemia and liver and kidney dysfunctions as evidenced by significant increase of RBCs count, HG content and some hematological parameters and significant decrease of AST, ALT, urea and creatinine levels. Histopathologically, edema and necrosis of kidneys, necrobiotic changes in brain, necrosis of liver, lymphoid depletion, hemorrhage of spleen, intermuscular hemorrhage with degeneration of cardiac muscles, consolidation of pulmonary alveoli in association with desquamation of intestinal villi and gastric mucosa were observed due to nitrite consumption. Histopathological changes were reduced due to addition of ascorbic acid and garlic oil. Consequently, using ascorbic acid and garlic oil alleviated the toxic hematological, serological and histopathological effects of sodium nitrite, but garlic oil was shown to ameliorate and correct such parameters close to the normal levels. In conclusion, garlic oil showed greater protective effect than ascorbic acid against nitrite toxicity.

Key words: Sodium nitrite • Ascorbic acid • Garlic oil • Hematological changes • Serological changes
• Histopathological changes

INTRODUCTION

Food additives are common and play an important role in our lives. Sodium nitrite (nitrite) has for decades been widely used for preservation of meat products and as an efficient inhibitor of *Clostridium botulinum* and thereby decreasing the risk of this organism producing toxins and heat-resistant spores. Nitrite also provides the processed meat with its characteristic red colour, flavours and aromas, known from products such as bacon and it inhibits lipid oxidation processes [1]. The wide use of nitrite in food technology elevates the importance of studying its effects on mammals [2, 3]. The hazardous effect of sodium nitrite is derived from the reaction of

nitrite with haemoglobin to form methaemoglobin which can reduce oxygen transport in the blood and the reaction of nitrite with secondary amines and amides in food under the acidic conditions of the stomach to produce nitrosamines and nitrosamides, respectively, in foods or in humans *in vivo*, which are considered to play vital roles in posing toxic effects in different organs [4]. Sodium nitrite was reported to cause cancer, hepatotoxicity, nephrotoxicity, dysregulation of inflammation and tissue injury [5]. Moreover, it was reported that prolonged use of sodium nitrite even within the permissible limits leads to anemia, liver and kidney dysfunctions, damage to brain cells in association with deleterious effect on male fertility [6].

Ascorbic acid is a naturally occurring organic compound and the main synthetic antioxidant used in meat industry; since it inhibits lipid peroxidation [7] and scavenges free radicals and reactive oxygen species [8] thereby spares vitamin E and protects the immune system [9]. In cured meats, ascorbic acid plays various roles; it can act as an effective reducing agent for a nitrite scavenger to extend the shelf life of meat products [10], as an enhancer of nitrosoheme pigments development and as an inhibitor of carcinogenic N-nitrosamine formation *in vitro* [11] and *in vivo* [12] by competing for the nitrosating agent. Thus, the presence of residual ascorbic acid allows nitrite to be reduced to nitric oxide, resulting in the gradual reduction of residual nitrite during storage.

Garlic (*Allium sativum L.*) clove is a good source of dietary phytochemicals and more than 30 organosulfur compounds with proven antibacterial [13], anticarcinogenic [14], hypolipidemic [15], hypoglycemic [16], antifungal [17], cardio protective, chemotherapeutic, antidiabetic and hepatoprotective [18], antiulcerogenic [19], anti-platelet aggregation, antibiotic, antidote for heavy metal poisoning [20] and antioxidant properties [21] protecting cell membranes and DNA from damage and several diseases [22], modulating the detoxification systems [20] and defending against free radicals damage, thereby preserving the body's healthy functioning [23]. Moreover, the beneficial effects of garlic and its constituents on neuronal physiology and brain function have begun to emerge in a wide range [24]. So, it's well recommended to use a moderate dose of garlic daily in children and adult's food since it protects them against pro-oxidant and other tragic effects resulting from chemical food additives. Additionally, garlic was particularly effective in reducing the chemical formation of N-nitrosomorpholine (NMOR) [25]. Meanwhile, there is a lack of experimental work concerning the protective effects of vitamins (ascorbic acid) and chelating agents (garlic oil) against the deleterious effect of nitrite as meat additive especially at the hematological, serological and histopathological levels [26].

Therefore, the aim of the present work was to prove the possibility to use a herbal medication such as "garlic oil" instead of pharmaceutical medication such as "ascorbic acid" via studying their protective roles against hematological, serological and histopathological effects of nitrite in male rats fed manually processed kofta supplemented with permissible limits of nitrite, ascorbic acid [2] and garlic oil 2% for 30-successive days.

MATERIALS AND METHODS

Animals: The present investigation was carried out on a total number of 20 apparently healthy adult male white albino rats (130-150 g body weight). These animals were housed for two weeks at constant environmental and nutritional conditions similar to those under which the experiment was performed for accommodation. Rats were housed in separate cages a way from any stressful stimuli and supplied with diet and water *ad libitum*.

Preservatives

Sodium Nitrite: Was incorporated in minced meat at concentration 125 ppm (permissible limit according to EOS [2]).

Ascorbic Acid: Was incorporated in minced meat at concentration of 500 ppm (permissible limit according to EOS [2]).

Garlic Oil 2%

Experimental Design: The male rats were divided randomly into 4 main groups (n=5) feeding on manually processed kofta supplemented with permissible limits of nitrite and ascorbic acid [2] and garlic oil 2% as follows:

Groups	Dose
Control	Control group received manually processed kofta without any treatment.
Group 1 (NO ₂)	Group fed on kofta supplemented with 125ppm sodium nitrite for 30 days.
Group 2 (NO ₂ + Ascorbic acid)	Group fed on kofta supplemented with 125 ppm sodium nitrite + 500 ppm ascorbic acid for 30 days.
Group 3 (NO ₂ + Garlic oil 2%)	Group fed on kofta supplemented with 125 ppm sodium nitrite + garlic oil 2% for 30 days.

At the end of the experimental period, overnight fasted animals were sacrificed by cervical dislocation and blood samples were collected in centrifuge tubes. Serum was and then quickly frozen at -20°C for biochemical analysis. Moreover, Specimens for histopathological examination were taken from different organs including liver, kidneys, lung, heart, spleen, brain, testes, stomach and intestine. Tests were performed in triplicate.

Hemogram Parameters: Complete blood picture was performed using HA-Vet Automatic Hematology analyzer (CLINDIAG SYSTEM).

Chemical Analysis of Serum:

AST and ALT: It were determined spectrophotometrically according to Reitman and Frankel [21].

Urea: It was determined spectrophotometrically according to Patton and Crouch [27].

Creatinine: It was determined spectrophotometrically according to Henery [28].

Histopathological Examination: Specimens from various organs (liver, kidneys, lung, heart, spleen, brain, testes, stomach and intestine) from the sacrificed animals of each group were collected and then fixed in 10% neutral buffered formalin. Following proper dehydration, clearing then the samples was embedded in paraffin wax. Sections about 5µm thickness were prepared and stained with H&E for histopathological examinations according to Drury and Willington [29].

According to the severity and dissemination of pathological alterations, the detected pathological lesions were graded qualitatively as following: Grade 0=normal histological texture, Grade 1= the detected pathological changes are at a mild degree; Grade 2= the identified pathological changes are at a moderate degree; Grade 3= the demonstrated pathological changes are at a severe degree [30].

Statistical Analysis: The data was statistically treated by one-way ANOVA using SPSS program for windows (Version 16) (SPSS Inc. Chicago, IL and USA) and Duncan's post hoc test with $P < 0.05$ considered significant. The results were expressed as means \pm SD.

RESULTS

Hematological Examination: The hematological results of sodium nitrite treated rats (group 1) showed significant decrease in RBCs count, HGB, WBCs, MCV, MCH, MCHC, lymphocytes and monocytes as compared to those of control animals (Table 1) revealing anemia during all the experimental duration. However, significant increase in such hematological parameters was observed in ascorbic acid and garlic oil 2% treated rats (groups 2 and 3) as compared to those of control group and nitrite treated animals group (1) (Table 1) revealing disappearance of anemia during all the experimental duration.

Serological Examination: With regard to the serum biochemical constituents in relation to liver and kidney functions of sodium nitrite treated groups (group 1), there were significant increase in the levels of AST, ALT, urea and creatinin levels (Table 2) revealing liver and kidney dysfunctions as proved by the histopathological alterations demonstrated in these organs. On the other hand, ascorbic acid and garlic oil 2% ameliorated the nitrite adverse effects as evidenced by a significant decrease of the serum activities of AST, ALT, urea and creatinine levels (Table 2).

Histopathological Examination

Liver: In-group 1, the liver exhibited dilatation and congestion of the central, portal veins and hepatic sinusoids with and activation of Von Kupffer's cells. Perivascular and peri-portal mononuclear leukocytic cellular infiltrations mainly lymphocytes and few

Table 1: The effect of permissible limit of sodium nitrite, ascorbic acid and garlic oil 2% on some hematological changes in male rats after 30 days

Group	Lymphocytes		Monocytes		HGB	RBCs*	WBCs ^c	MCV*	MCH ^c	MCHC ^c
	%	No. ^a	% ^a	No. ^a						
Control	9.07±0.43 ^a	0.68±0.08 ^b	2.48±0.13 ^b	0.22±0.02 ^a	29.08±1.17 ^b	7.37±0.32 ^b	7.52±0.10 ^b	55.00±1.87 ^c	37.59±0.78 ^c	65.86±1.33 ^{ab}
Group 1 (NO ₂)	9.07±0.19 ^a	0.57±0.60 ^a	2.41±0.12 ^b	0.21±0.01 ^a	28.66±0.83 ^b	6.74±0.35 ^c	6.18±0.33 ^c	54.40±4.10 ^c	37.40±1.26 ^c	63.22±2.52 ^b
Group 2 (NO ₂ + Ascorbic acid)	9.24±0.51 ^a	0.81±0.06 ^c	2.67±0.08 ^a	0.20±0.03 ^a	29.12±1.47 ^{ab}	7.76±0.24 ^b	8.90±0.90 ^a	61.20±6.10 ^b	39.60±2.25 ^b	67.88±1.42 ^a
Group 3 (NO ₂ + Garlic oil 2%)	9.35±0.61 ^a	0.83±0.07 ^c	2.69±0.12 ^a	0.16±0.01 ^b	30.86±1.60 ^a	8.27±0.43 ^a	8.94±0.80 ^a	66.80±2.95 ^a	42.70±1.50 ^a	68.18±3.15 ^a

The values represent Mean \pm SD of three experiments.

Means within a column followed by different letters are significantly different ($P < 0.05$)

Table 2: The effect of permissible limit of sodium nitrite, ascorbic acid and garlic oil 2% on some liver and kidney function tests in male rats after 30 days

Groups	AST	ALT	Urea	Creatinine
Control	354.68±59.48 ^a	75.42±24.61 ^{ab}	39.44±5.77 ^c	0.52±0.11 ^a
Group 1 (NO ₂)	365.26±27.03 ^a	89.42±17.63 ^a	60.92±5.78 ^a	0.58±0.04 ^a
Group 2 (NO ₂ + Ascorbic acid)	317.74±32.24 ^{ab}	58.32±3.61 ^b	50.72±4.61 ^b	0.52±0.11 ^a
Group 3 (NO ₂ + Garlic oil 2%)	252.48±66.30 ^b	57.96±18.81 ^b	50.40±9.26 ^b	0.46±0.09 ^a

The values represent Mean \pm SD of three experiments.

Means within a column followed by different letters are significantly different ($P < 0.05$).

Table 3: Summary of the histopathological lesions in different treated groups

Lesion score	Treatment		
	Group 1	Group 2	Group 3
Liver			
Congestion of hepatic blood vessels	3	2	1
Activation of Von Kupffer cell	2	1	1
Perivascular leukocytic infiltration	2	1	0
Degenerative changes	3	2	1
Necrosis of hepatic cells	3	1	0
Nuclear changes	3	1	0
Hyperplasia of bile duct	2	1	0
Kidney			
Congestion of renal blood vessels	3	2	1
Degeneration of blood vessels wall	2	1	0
Perivascular edema	2	1	0
Tubular epithelial degeneration and necrosis	3	2	1
Hyaline and cellular casts	3	2	1
Interstitial leukocytic infiltration	2	1	0
Cystic dilatation of renal tubules	2	1	1
Shrinkage of glomerular tuft	2	1	1
Lung			
Perivascular edema	3	1	0
Peribronchial leukocytic aggregation	3	2	1
Consolidated alveoli	3	1	0
Intestine			
Enteritis	3	2	0
Leukocytic infiltrations in sub mucosa	2	1	0
Brain			
Congestion of blood vessels	3	2	1
Neuronal degeneration	3	2	1
Hemorrhage	3	2	1
Encephalomalacia	3	1	0
Perineuronal edema	2	1	0
cellular infiltrations	2	2	1
Spleen			
Hemorrhage	3	2	1
lymphoid depletion	2	2	1
Apoptosis	2	1	0
Thickening and hyalinization of blood vessels	2	1	0
Macrophage cell infiltrations	3	1	1
Lymphoid hyperplasia	0	0	2
Heart			
Congestion of myocardial blood vessels	3	2	1
Inter-muscular hemorrhage	3	1	0
Hyaline degeneration of cardiac muscle	2	1	0
Testes			
Inter-tubular edema	2	1	0
Destruction of basement membrane of seminiferous tubules (SNT)	2	0	0
Degeneration of lining epithelial cells of seminiferous tubules (SNT)	3	2	1
Vacuolation in the SNT lumen	3	2	1
Necrosis of spermatogenic cells	2	1	0
Stomach			
Congestion and dilatation of blood vessels	3	2	1
Desquamation of the mucosal lining epithelium	3	1	0
focal mononuclear cellular aggregation in the tunica muscularis	2	1	0

Group 1 (N2 group), Group 2 (N2+Ascorbic acid group), Group 3 (N2+garlic group), (0), lesions not observed; (1), mild lesion; (2), moderate lesion and (3), severe lesion

macrophages accompanied by disruptions of the hepatic cords are seen (Fig. 1A). Hyperplasia of the lining epithelial cells of bile duct with newly formed bile ductules (Fig. 1B) as well as proliferation of fibrous connective tissue around the portal area was also demonstrated. Furthermore, marked diffuse hydropic degeneration of the hepatocytes characterized by swollen, pale, vacuolated cytoplasm with pyknotic nuclei (Fig. 1C). In addition, focal areas of lytic necrosis characterized by disappearance of hepatocytes and replaced by erythrocytes were detected displaced the hepatic parenchyma (Fig. 1D).

In-group 2 (nitrite plus ascorbic acid), congestion of the central and portal veins; the hepatic sinusoids were also dilated and congested with enlargement and activation of Von Kupffer's cells (Fig. 1E). Rarely, the portal areas were expanded by few numbers of lymphocytes. Multifocally, the hepatocytes showed hydropic degeneration characterized by indistinct vacuolation of the cytoplasm mainly in centrilobular zones of hepatic lobules; while the hepatocytes in the peripheral zone of hepatic lobules showed normal histological appearance.

Interestingly, in-group 3 (nitrite plus garlic), only congestion of central veins (Fig. 1F) with occasional activation of Von Kupffer's cells was observed. However, the hepatic tissue showed almost intact hepatic cords.

Kidneys: In-group 1, congestion of the renal blood vessels and intertubular capillaries was seen. The perivascular interstitium in renal cortex expanded by oedema and admixed with erythrocytes and few inflammatory cells with marked hypertrophy and vacuolization of muscular blood vessels wall were demonstrated (Fig. 2A). Periglomerular and intertubular hemorrhage was also observed. Multifocally, the lining epithelium of large numbers of renal convoluted tubules showed marked vacuolar and hydropic degeneration characterized by swollen pale vacuolated cytoplasm and indistinct cell borders. Occasionally, necrosis of the tubular epithelium was characterized by highly eosinophilic cytoplasm with shrunken, pyknotic nuclei and loss of cellular details (Fig. 2B) resulting in destruction and desquamation of epithelial lining in their lumen. Accordingly, most renal tubules contained hyalinized eosinophilic and cellular renal casts in their lumen (Fig. 2C). Rarely, interstitial lymphocytic cellular infiltration was seen in few cases in-between the renal tubules. Moreover, some renal tubules in both cortex and medulla exhibited cystic dilatation and lined by attenuated

epithelium. Multifocally, in renal cortex some glomeruli showed shrinkage of glomerular tufts with coagulative necrosis of the lining cells of some glomerular tufts and renal tubules characterized by pyknosis of the nuclei and more eosinophilic cytoplasm with retention of tissue architecture (Fig. 2D).

In-group 2, mild congestion of the renal blood vessels and intertubular capillaries was noticed. The lining epithelium of some proximal and distal convoluted tubules in renal cortex appeared less degenerated and exhibited mild vacuolar and hydropic degeneration. Additionally, the hyaline casts were also infiltrated in few cases (Fig. 2E). Mild mononuclear cell infiltration was detected in the interstitium. Multifocally, the cortical interstitium, predominantly around cortical blood vessels and glomeruli was occasionally expanded by oedema admixed with erythrocytes and few inflammatory cells.

In-group 3, mild tubular changes with mild congestion of the renal blood vessels and intertubular capillaries were seen. Interestingly, mild degeneration of the lining epithelium of renal tubules in combination with normal histological structure of glomeruli was also noticed (Fig. 2F).

Lung: In group 1, congested blood vessels with perivascular edema admixed with inflammatory cellular infiltration mainly lymphocytes and macrophages were noticed. The lungs showed marked peribronchial lymphocytic cellular aggregation that displaced part of bronchiolar and alveolar architecture. Moreover, the alveoli were markedly consolidated with eosinophilic debris and occasionally admixed with macrophages, lymphocytes and erythrocytes and alternated with emphysema (Fig. 3A and B). On the other hand, congestion of interalveolar capillaries in association with few alveoli containing eosinophilic debris (Fig. 3C) was seen in-group 2. Few peri-bronchial and perivascular mononuclear infiltrations were observed. Conversely, there are no clear pathological changes noticed in the pulmonary tissues of group 3 except congestion of interalveolar blood capillaries with few peribronchial mononuclear leukocytic infiltration (Fig. 3D).

Intestine: The microscopic examination of the intestines of rats of group 1 revealed severe enteritis characterized by thickening of the intestinal mucosa with necrosis of the lining epithelium of intestinal villi with inflammatory cellular infiltrations mainly lymphocytes and macrophages in the lamina propria (Fig. 3E). Variably sized aggregates

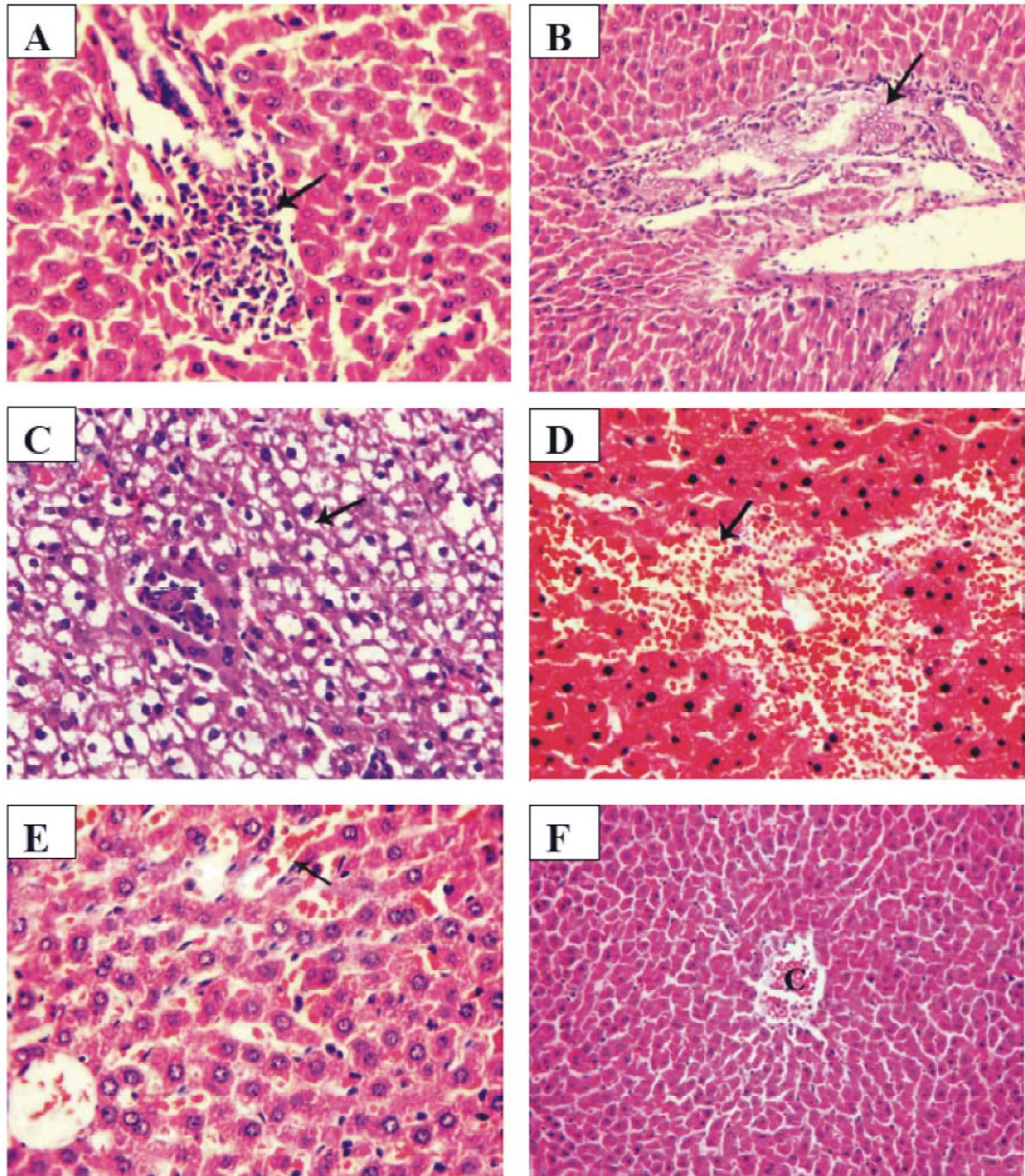


Fig. 1: H&E stained section of liver of group 1 (A-D) showing:

- (A) periportal leukocytic cellular infiltration mainly lymphocytes displaced the hepatic parenchyma (arrow, x 400)
- (B) hyperplasia of the lining epithelium of the bile ducts (arrow, x 200)
- (C) marked hydropic degeneration of the hepatocytes characterized by swollen, pale, vacuolated cytoplasm with pyknosis (x 400)
- (D) focal area of lytic necrosis with replacement of vacant space by aggregates of erythrocytes (asterisk, x 200)
- (E) liver of group 2 showing dilated and congested hepatic sinusoids with enlargement and activation of Von Kupffer's cells (arrow, x 400)
- (F) liver of group 3 showing congestion of the central veins (C) with normal histological structure of hepatocytes (x 100)

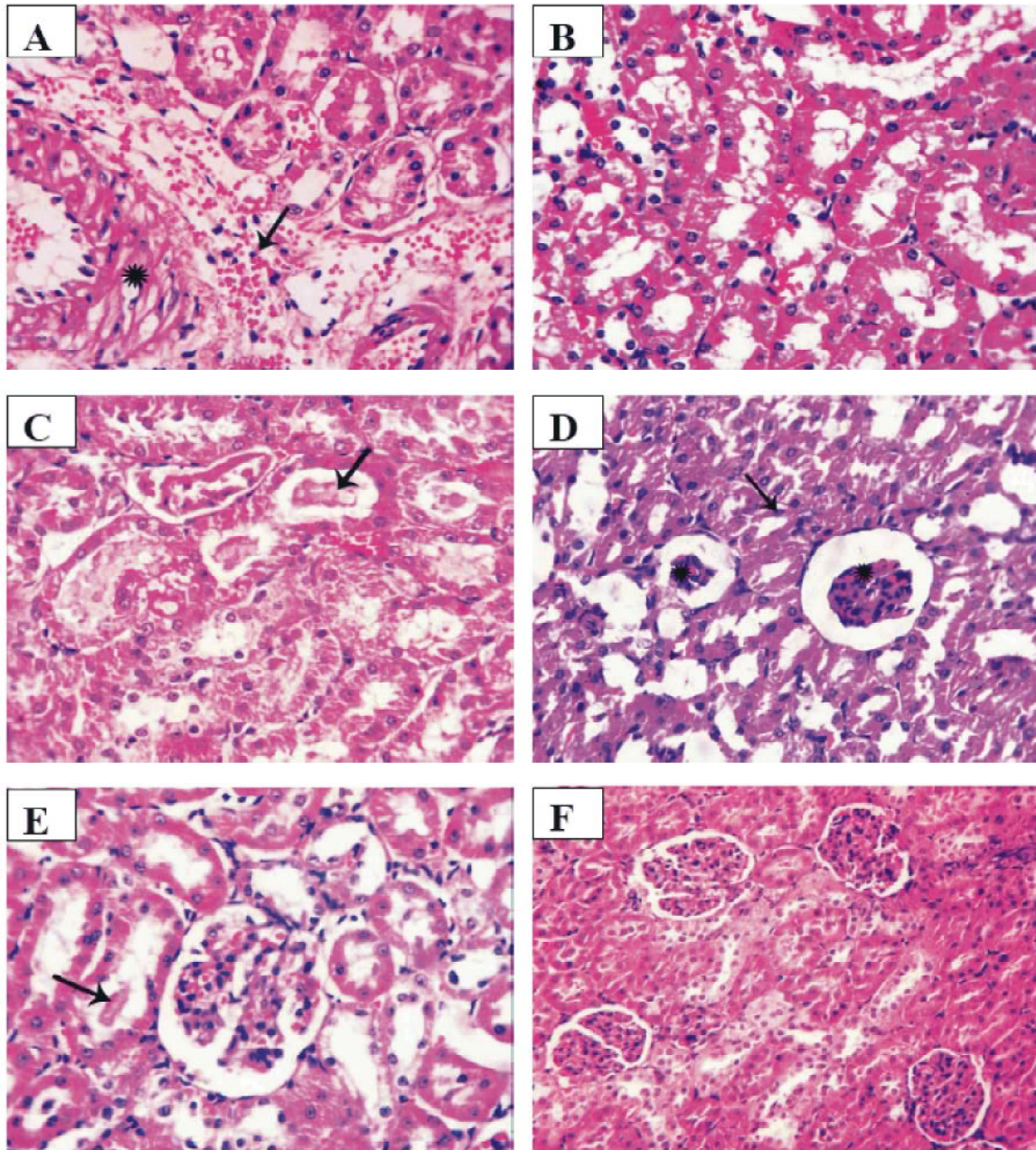


Fig. 2: Kidney section stained with H&E of group 1 (A-D) showing:

- (A) perivascular interstitium in renal cortex expanded by edema admixed with erythrocytes and few inflammatory cells (arrow) with degeneration and vacuolation of blood vessels wall (asterisk, x 400)
- (B) necrosis of the lining epithelium of some proximal and distal convoluted tubules (x 200)
- (C) cellular and homogenous eosinophilic casts in the lumen of some renal tubules (arrow, x 400)
- (D) shrinkage of the glomerular tuft with coagulative necrosis of the lining cells of some glomerular tufts(asterisk) and renal tubules (arrow, x 200)
- (E) kidney of group 2 showing congestion of the glomerular capillaries with the presence of few eosinophilic cellular casts (arrow) in the lumen of some renal tubules (x 200)
- (F) kidney of group 3 showing mild degeneration of the lining epithelium of renal tubules(x 100)

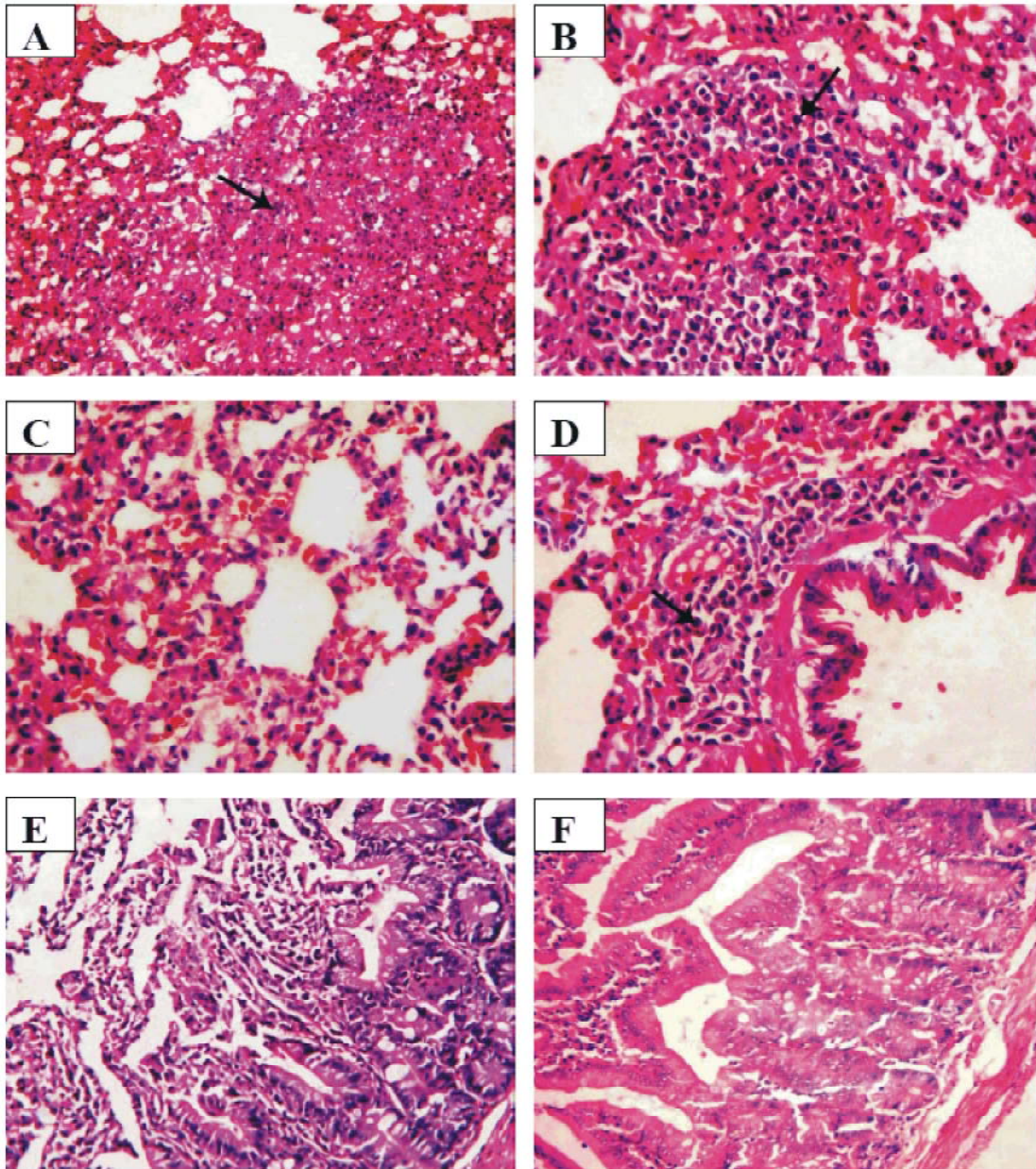


Fig. 3: Lung section stained with H&E of group 1 (A-B), of group 2 (C), of group 3 (D) and intestine section stained with H&E of group 1 (E) and group 3 (F) showing:

- (A) focal areas of alveolar consolidation (arrow) filed with erythrocytes admixed with inflammatory cells and alternated with emphysema (x 200)
- (B) consolidated alveoli filled with aggregation of mononuclear inflammatory macrophages and lymphocytes (arrow, x 400)
- (C) congestion of interalveolar capillaries with consolidation of few alveoli (x 200)
- (D) few peribronchial mononuclear leukocytic infiltration (arrow, x 400)
- (E) Intestine of group 1 showing necrosis of the lining epithelium of intestinal villi with inflammatory cellular infiltrates in the lamina propria. H&E stain, x200
- (F) Intestine of group 3 showing nearly normal histological architecture of intestinal mucosa. H&E stain x400

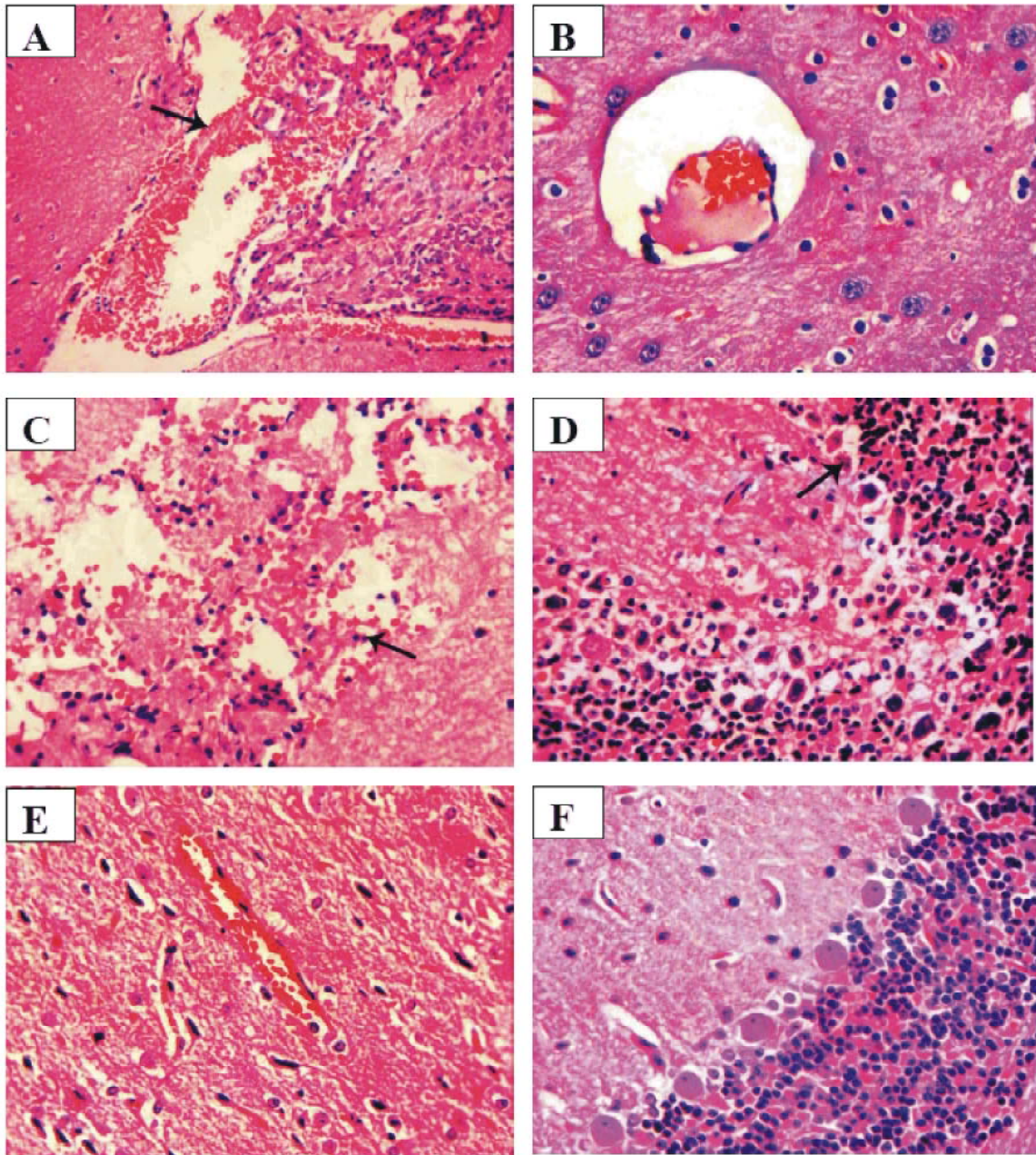


Fig. 4: H&E stained section of brain of group 1 (A-D), group 2 (E) and group 3 (F) showing:

- (A) extensive hemorrhages in the ventricles (x 200)
- (B) thrombosis of cerebral blood vessels with dilatation of Virchow Robin spaces, notice also perineuronal vacuolation (x 200)
- (C) diffuse area of malacia characterized by abundant clear spaces infiltrated by few numbers of erythrocytes and lymphocytes (arrow, x 200)
- (D) neuronal degeneration (arrow) with swollen rounded cell bodies and indistinct or eccentric pyknotic nucleus. Notice also, area of malacia (asterisk, x 200)
- (E) Brain of group 2 showing mild congestion of cerebellar blood vessels with elongation of its endothelial lining (x 200)
- (F) Brain of group 3 showing nearly normal purkinje neurons of cerebellum (x 200)

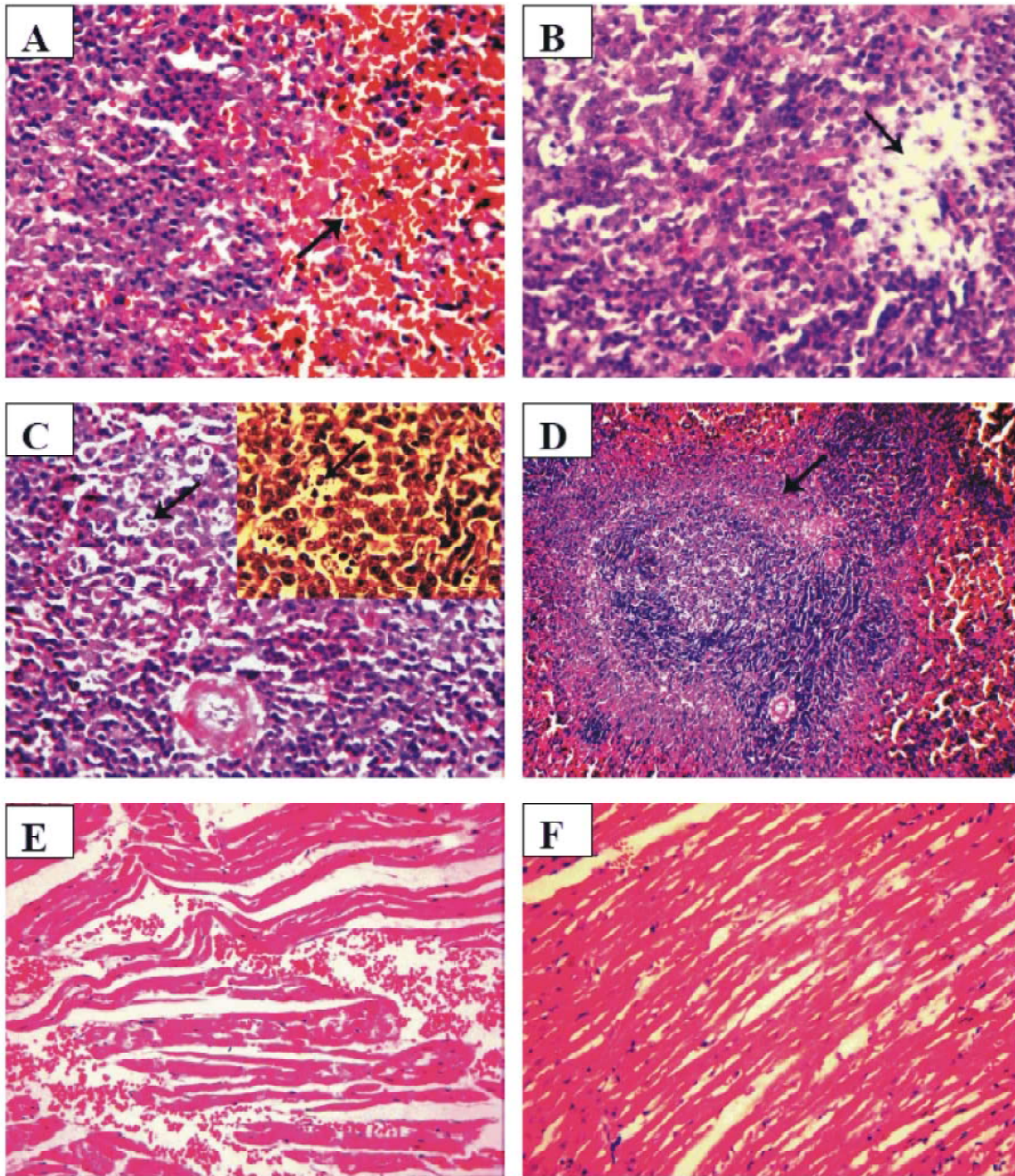


Fig. 5: Spleen section stained with H&E of group 1 (A-C), and figure (D) of group 3 and heart section stained with H&E of group 1 (E), and of group 3 (F) showing

- (A) focal areas of hemorrhages in the red pulp (x 400)
- (B) lymphoid depletion of some lymphoid follicles of the white pulp (arrow, x 400)
- (C) lymphoid depletion of the white pulp with apoptosis of some lymphoid cells (arrow, x 400, inset x 1000).
- (D) proliferation of the lymphoid elements of white pulp (arrow, x 100)
- (E) heart of group 1 showing intermuscular hemorrhage (x 200)
- (F) heart of group 3 showing normal architecture of cardiac muscles (x 200)

of sloughed enterocytes admixed with eosinophilic cellular and karyorrhectic debris was seen in the intestinal lumen. Moreover, the sub mucosa showed congested blood vessels and infiltrated by small numbers of inflammatory cells. Meanwhile, congestion of blood vessels with mild desquamation of intestinal villi in association with few inflammatory cells in the lamina propria was observed in rats of group 2. On the other side, mild congestion of mucosal blood vessels with normal histological structure of intestinal villi (Fig. 3F) was noticed in rats of group 3.

Brain: In group 1, marked congestion of meningeal, cerebral and cerebellar blood vessels and diffuse hemorrhage in the cerebellum as well as in meninges were observed in brain tissues. Furthermore, noticeable congestion of choroid plexus and perivascular hemorrhage in the ventricles (Fig. 4A) was observed. Multifocal hemorrhages were also detected in different sites particularly brain stem. Thrombosis of cerebral blood vessels with dilatation of Virchow Robin spaces with perineuronal vacuolation (Fig. 4B). Moreover, focal areas of encephalomalacia infiltrated with erythrocytes leukocytic cells mainly lymphocytes was demonstrated (Fig. 4C). Focal areas of gliosis in the brain tissue were also seen. Area of rarefaction predominantly affecting the gray matter of cerebellum with necrosis of Purkinje cell that take angular shape, deeply eosinophilic cytoplasm and pyknosis or absent nuclei (Fig. 4D) was also noticed. Furthermore, the brain showed numerous microglia cells around the degenerated neurons manifested by micro gliosis. Neurophagia was characterized by engulfing of microglia cells to the necrotic neurons. Perineuronal edema and vacuolation were also noticed around the degenerated neurons. In group 2, the neurons exhibited mild degenerative and necrobiotic changes with focal area of hemorrhage in the brain tissues of few cases. Mild congestion of cerebellar blood vessels with elongation of its endothelial lining (Fig. 4E) was detected. In-group 3, the neurons displayed no prominent pathological change as the cerebral tissues revealed nearly normal purkinje neurons of cerebellum in most examined cases (Fig. 4F).

Spleen: The red pulp of spleen of rats in-group 1 exhibited congested blood vessels and sinusoids with extensive areas of hemorrhages (Fig. 5A). Additionally, the interstitium was expanded by aggregates of mononuclear inflammatory cells mainly lymphocytes and macrophages. Furthermore, marked lymphoid depletion was

demonstrated in some lymphoid follicles of the white pulp accompanied by apoptosis of some lymphoid cells was commonly observed (Fig. 5B and C). Occasionally, thickening and hyalinization of the vascular wall of central artery was also demonstrated in some examined cases.

In-group 2, congested blood vessels and sinusoids of the red pulp was noticed. Accidentally, mild lymphoid depletion of the white pulp accompanied by apoptosis of some lymphoid cells was also seen in some cases. Meanwhile, in-group 3, mild congestion of blood vessels of red pulp with lymphoid hyperplasia of the lymphoid follicles of white pulp was demonstrated (Fig. 5D) with absence of apoptosis of lymphocytes.

Heart: Marked congestion of myocardial blood vessels was the main pathological changes detected in rats of group 1 with extensive hemorrhages in-between the cardiac muscle fibers was observed (Fig. 5E). Additionally, hyaline degeneration of cardiac muscle fibers was also noticed in some cases. Furthermore, degeneration of myocytes characterized by discrete clear sarcoplasmic vacuoles that flattened and displaced the nucleus to the periphery was also observed in some cases.

In-group 2, Congestion of myocardial blood vessels was detected. Rarely, intermuscular hemorrhages admixed with few numbers of lymphocytes and macrophages. Accidentally, hyaline degeneration of cardiac muscles was seen in two examined cases. On the other side, mild congestion of myocardial blood vessels was the main pathological alterations observed in rats of group 3. Interestingly, the cardiac muscles were nearly similar to control group (Fig. 5F).

Testes: H & E stained sections of the testis of rats of group 1, displayed congestion of testicular blood vessels with intertubular edema. However, entire degeneration and necrosis of the lining epithelial cells of some seminiferous tubules was detected (Fig. 6A) with incomplete spermatogenesis and absence of spermatozoa in the lumen of these degenerated tubules. Destruction of the basement membrane of some seminiferous tubules was also detected and accumulation of edematous fluid in the lumen of some seminiferous tubules was seen. Meanwhile, degenerative changes of germ cells lining seminiferous tubules characterized by swollen, pale and vacuolated cytoplasm (Fig. 6B) were observed in rats of group 2. However, the testes of rats of group 3 showed nearly normal histological architecture (Fig. 6C).

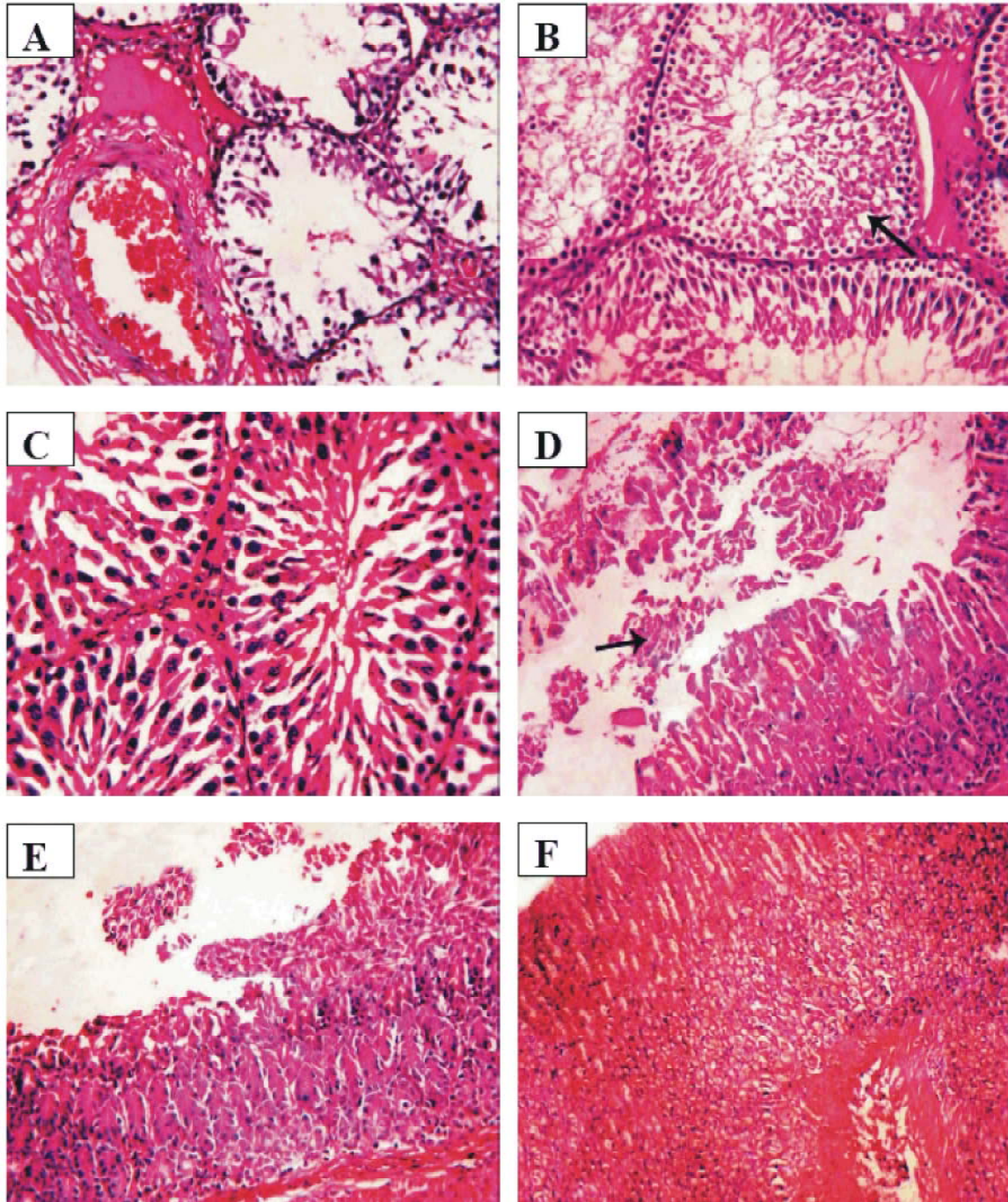


Fig. 6: Showing the following:

- (A) Testes of group 1 showing necrosis of germ cells lining of seminiferous tubules (x 200)
- (B) testes of group 2 showing swollen, pale and vacuolated germ cells lining seminiferous tubules (arrow, x 200)
- (C) Testes of group 3 showing normal spermatogenic cells lining of seminiferous tubules (x 400)
- (D) Stomach of group 1 showing desquamation of the lining epithelium of gastric mucosa (arrow, x 200)
- (E) Stomach of group 2 showing mild desquamation of the lining epithelium of gastric mucosa (x 200)
- (F) Stomach of group 3 showing normal histological structure of gastric mucosa (x 100)

Stomach: Stomach of rats in group 1 showed congestion of mucosal blood vessels. Additionally, desquamation of the lining epithelium (Fig. 6D) with focal mononuclear cellular aggregation in the tunica muscularis was demonstrated.

Meanwhile, in-group 2, mild congestion of mucosal blood vessels in association with desquamation of the lining epithelium of gastric mucosa was observed (Fig. 6E). Interestingly, nearly normal histological structure of gastric mucosa (Fig. 6F) with mild congestion of the gastric blood vessels was detected in stomach of rats of group 3.

DISCUSSION

Natural and synthetic food additives are used to maintain or improve the safety, nutrient value, taste and texture of food. Sodium nitrite and other additives may react with amines of food in the stomach and produce nitrosamines and free radicals. Such products can be harmful to different organs [31]. In addition, dietary intake of naturally occurring antioxidants may be the most sensible means to prevent biochemical alterations and disease risk factors associated with free radical formation.

In the present study, the hematological results of nitrite treated rats (group 1) agree with those of Elsabagh *et al.* [6] and Helal and Abdel-Rahman [32].

In the present work, anemia in nitrite treated rats (group 1) could be attributed to slowing down or obstructing the NAD and NADP reduction processes in erythrocytes by nitrite ions resulting in disturbing their cell respiration, lysis of RBCs and breakdown of HGB to bilirubin in the liver [33] and earlier removal of erythrocytes from circulation, thus shortening their life cycle [34].

Additionally, the significant decrease in HGB concentration in blood of rats administered sodium nitrite has been shown to be a result of nitrites induced disturbances in heme biosynthesis [35] and nitrites induced conversion of ferrous ion of HGB to ferric ion both *in vivo* and *in vitro* [36]. Generally, adverse changes in values of the erythrocytic system markers, occurring under the influence of sodium nitrite, may be possibly through disorders of erythrocyte synthesis, enhanced hemolysis and water shifting to the extracellular compartment [37].

In the current study, the serological results in relation to liver and kidney functions of nitrite treated group (group 1) agree with those of Elsabagh *et al.* [6] and Hassan - Hanan *et al.* [38].

The increase in the activity of AST and ALT enzymes in the serum of nitrite treated rats could be attributed to the formation of over 300 cytotoxic N-nitrosocompounds and free radicals, in the acidic environment of the stomach as a result of combination of sodium nitrite with secondary amines in the food or the body, causing lipid peroxidation and severe hepatic and renal necrosis [39] as confirmed by the obtained results of histopathological examination that revealed damage of hepatic tissues, or may be due to anemia and methemoglobinemia which induced hypoxic injury to centrilobular hepatocytes that consequently cause enzyme leakage [40].

Furthermore, in response to nitrite treatment, urea and creatinine increased in the serum, suggesting an impairment of kidney functions. These effects could also be attributed to the cytotoxic effect of N-nitroso compounds in renal tubular cells and the changes in the threshold of tubular reabsorption, renal blood flow and glomerular filtration rate [41]. The obtained histopathological results that revealed damage of renal tissues coincided with the obtained biochemical findings.

Meanwhile, necrosis and apoptosis in various investigated organs (especially brain) following nitrite supplementation could be attributed to inhibition of ATP production by nitrite resulting in cellular energy deficiency and brain damage [42], or due to the increased nitric oxide (NO) in tissues that may be resulted from the energy depletion of mitochondrial phosphorylation (ATP) [43]. These changes could be attributed to the role of nitrite in the induction of oxidative stress in brain of rat resulting in inhibition of neuron activities as it produced some neuro biochemical alterations in the brain tissues such as inhibition of Acetylcholine esterase (AChE) and elevation of lactate dehydrogenase (LDH) activity [44]. These enzymes have been used as standard biomarkers of toxic stress [45]. The decreased AChE activity could be due to a decrease of the enzyme synthesis by the inhibitory action of the toxicants [45]. Accordingly, its inhibition could decrease cellular metabolism, induce deformities of cell membrane, differential membrane permeability, ionic refluxes and disturb metabolic and nervous activity [46]. On the other hand, LDH is involved in energy production and the elevation of LDH activity might play a role in brain damage after treatment with nitrite [47]. This elevation could be attributed to a generalized increase in membrane activity due to the increase of one of isoenzymes of LDH [48].

Furthermore, degeneration and necrosis of some of the spermatogenic cells in association with other pathological changes in the testicular tissues of nitrite

treated rats could be due to defect in the function of Sertoli cell with consistent lipid peroxidation (LPO) and altered membrane properties that led to germ cell death at different stage [49].

Moreover, various histopathological lesions in various organs in the present work are mainly attributed to oxidative stress where the nitrite interacted with mitochondria, peroxisomes and microsomes, leading to excessive generation of ROS that capable of depleting endogenous antioxidant status and causing oxidative damage to biomolecules such as membrane lipids, DNA and a variety of transport proteins, including Na⁺/K⁺-ATPase, which results in cell-death and organ-dysfunction [41]. Additionally, production of reactive nitrogen species by nitrite plays an important role in its carcinogenic effect through its reaction with body tissues and triggering lipid peroxidation, DNA lesions, enzyme inactivation and damage of different organs [50].

In the current work, the administration of ascorbic acid and garlic oil variably improved hepatic and kidney functions as evidenced by hematological, serological and histopathological findings. However, the mechanism by which ascorbate protects rats from deleterious effect of sodium nitrite is not completely understood. Recently, Mirvisch *et al.* [11] demonstrated that ascorbate blocks the formation of nitrosamines *in vitro* by competing for available nitrite. Ascorbic acid provided a moderate degree of protection and improvement against nitrite due to its ability to interact with critical biomolecules and neutralizing free radicals resulting in decreasing its hepatic and renal burdens [51], in addition to protection of the cells from expansion or abnormalities in their structural features [52].

The obtained results in the present study revealed that the administration of garlic oil with sodium nitrite markedly reduced the severity of pathological alterations as well as the hepatic and renal functions that induced by nitrite only. Moreover, the serological results in relation to liver and kidney functions of garlic oil treated rats (group 3) in the present study agree with those of Bhattacharjee *et al.* [52], Hassan *et al.* [53] and Abdul-Ameer and Abed [54].

These findings confirmed that administration of garlic oil has an extremely beneficial role in overcoming the occurred adverse effects of sodium nitrite, which is probably due to its excellent antioxidant properties [38], which may eventually be related with the preservation of Super oxide dismutase (SOD) activity, primary mitochondrial role against nitrite-induced neurotoxicity in rats, reducing the chemical formation of nitroso amines [55] and the ability of Diallyl disulfide and Diallyltrisulfide

present in garlic oil in modulating the oxidative stress and detoxifying enzyme system [56]. Allyl sulfur compounds in garlic juice might also act as nitrite scavengers [55].

Generally, the antioxidant efficiency of garlic oil is due to the presence of natural organosulfur compounds, such as allicin, alliin, S-allyl cysteine, allyl disulfide and diallyl disulfide, which elevate the levels of antioxidant enzymes (SOD, CAT and GPx) that can neutralize several types of free reactive radicals such as reactive oxygen species (ROS) which induce oxidative stress and cellular injury [50]. These biologically active organosulfur compounds can easily infuse phospholipid membranes and reduce intracellular nitrite to enhance its excretion from the body, resulting in reduced nitrite accumulation in tissues and blood, thus, providing protection against free radical damage in the body [57]. However, garlic oil plays an important role in stabilizing the cell membrane and its ability to reduce or prevent lipid peroxidation resulting in protection of the liver from free radical-mediated hepatic and renal tissues toxicity [58-59]. Meanwhile, the beneficial effects of garlic oil intake against the pathological alterations in the brain tissues appeared through the significant restoration of cellular enzymes; AChE and LDH. These results may be attributed to the ability of garlic oil in protecting the brain tissues from free radical-mediated toxic damages. The garlic oil effect depends upon stabilizing cell membrane permeability and maintenance the normal levels of the membrane bound enzymes [24].

CONCLUSIONS

Garlic oil, as a natural food additive, ameliorated to a higher extent than ascorbic acid, the anemia, the impairment of liver and kidney functions, damage to brain cells in association with deleterious effect on male fertility as well as the pathological changes in lung, heart, stomach, intestine and spleen in male rats that ingested sodium nitrite even within the permissible limits. Consequently, it is recommended to use garlic oil as a protective agent for the toxic effects of sodium nitrite.

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