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Amoxicillin–clavulanic acid induced sperm abnormalities and histopathological changes in mice

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ABSTRACT

Objective: To explore the genotoxic potential and histopathological changes induced in liver, kidney, testis, brain and heart after using the antibiotic drug amoxicillin/clavulanic acid (4:1).

Methods: The study included chromosomal aberration analysis in bone-marrow and mouse spermatocytes, induction of sperm morphological abnormalities and histopathological changes in different body organs. The drug was administrated orally at a dose of 81 mg/kg body weight twice daily (Total = 162 mg/kg/day) for various periods of time equivalent to 625 mg/men (twice daily).

Results: The results revealed non-significant chromosomal aberrations induced after treatment with amoxicillin/clavulanic acid (AC) in both bone marrow and mouse spermatocytes after 7 and 10 days treatment. On the other hand, statistically significant percentages of sperm morphological abnormalities were recorded. Such percentage reached 8.10 ± 0.55, 9.86 ± 0.63 and 12.12 ± 0.58 at the three time intervals tested (7, 14 and 35 days after the 1st treatment respectively) (treatment performed for 5 successive days) compared with 2.78 ± 0.48 for the control. The results also revealed histopathological changes in different body organs after AC treatment which increased with the prolongation of the period of therapy. Congestion of central vain, liver hemorrhage and hydropic changes in hepatocytes were noticed in the liver. Degenerative changes were found in kidney glomerulus and tubules while testis showed atrophy of seminiferous tubules, and reduction of spermatogenesis. AC also induced neurotoxicity and altered brain neurotransmitter levels. Hemorrhage in the myocardium, disruption of cardiac muscle fibers and pyknotic nuclei in cardiomyocytes were recorded as side effects of AC in heart tissue.

Conclusions: The results concluded that AC treatment induced sperm morphological abnormalities and histopathological changes in different body organs. Clinicians must be aware of such results while describing the drug.

1. Introduction

Combination of amoxicillin–clavulanic acid (co-amoxyclav) was considered as critical and broad spectrum drugs for human and animal health by the World Health Organization. Amoxicillin is an antibiotic in a group of drugs called penicillin. Amoxicillin–clavulanic acid (AC) is a commonly prescribed drug for bacterial

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infections such as sinusitis/otitis [1], pneumonia [2], bacterial bronchitis [3], ear and skin infections [4]. Microbiological, pharmacological, and clinical studies show that clavulanic acid in combination with amoxicillin is compatible, efficient and safe in the treatment of urinary tract infections caused by amoxicillin-resistant pathogens. The activity spectrum is extended to B-lactamase forming organisms, surgical antimicrobial prophylaxis and post-operative infections in urology [5,6]. The combination of amoxicillin–clavulanic acid and ketoconazole treatment is recommended to treat eumycetoma patients with *Staphylococcus aureus* co-infection [7]. AC was also used in *Helicobacter pylori* eradication [8].

Amoxicillin and the similar antibiotics act by inhibiting the formation of bacterial cell wall. Clavulanic acid is found in a class of medications called beta-lactamase inhibitors, which works by preventing bacteria from destroying amoxicillin [9].

The safety of antibiotics is becoming a worldwide concern. The studies concerning the effect of amoxicillin on the genetic material are rare. Li *et al*. [9] reported that amoxicillin induced intracellular reactive oxygen species (ROS) at the tempo similar to that of DNA lesions induction. Oxidative DNA damage in mammalian cells via ROS was also reported by other authors [8]. Li *et al*. [9] also indicated that DNA lesions were resulted from amoxicillin oxidative damage on DNA nucleotides purines and pyrimidines. Such amoxicillin-induced DNA lesions can be repaired quite effectively within a short period after administration of the drug. On the contrary, the potential genotoxic effect of amoxicillin was investigated *in vitro* in human peripheral lymphocytes by sister chromatid exchange (SCE), chromosomal aberration (CA) and micronucleus (MN) test [10]. The authors suggested that Amoxicillin did not pose a genotoxic risk to patients who were under therapy against bacterial infection. Many authors recorded the hepatic side effect after treatment with AC [11–14]. Evidences of oxidative stress, renal damage [15], and altered brain neurotransmitter were also recorded [16].

The present investigation has been made to assess the genotoxicity of AC in somatic and germ cells of male mice using chromosomal aberration analysis in bone marrow and mouse spermatocytes and morphological sperm abnormalities as markers for genotoxicity and infertility. Histopathological investigations concerning its effect in the tissues of liver, kidney, testis, brain and heart were also performed.

2. Materials and methods

2.1. Chemical drug

The antibiotic drug Hibiotic which consists of amoxicillin–clavulanic acid (4:1) was tested in this study. The drug is manufactured by Amoun Pharmaceutical Co. SAE El-Obour City, Cairo, Egypt. M.O.H. Reg. No. 20852/2012.

2.2. Doses

The recommended therapeutic doses for men are 375, 625 mg and 1 g twice daily. The tested dose for mice is 81 mg/kg b. wt. (162 mg/kg/day) administrated orally twice daily. Such dose is equivalent to 625 mg/men (twice daily) after modification by relative surface area between species according to the Paget and Barnes formula [17].

2.3. Experimental animals

Male Swiss Albino mice (*Mus musculus*) three months old weighing about 25 g were obtained from the animal-house colony of the National Research Centre (Dokki, Cairo, Egypt). The animals were housed in stainless steel wire mesh cages on a bedding of wood shavings. Ambient temperature was controlled at (25 ± 3) °C with relative humidity of 50% ± 15% and a light/dark cycle of 12 h/12 h. Food and water were provided *ad-libitum*.

2.4. Ethics

The handling of animals and the experimental protocol all followed the Ethics of animal care and Ethical Committee of the National Research Centre, and performed for being sure that animals do not suffer at any stage of the experiments.

2.5. Experimental design

After one week of acclimation in the lab, the animals were fasted overnight before treatment and then divided randomly into the following groups:

Group I: Control (untreated). Group II: In this group, animals were tested for chromosomal aberration analysis and histopathological evaluation. The group (II) was subdivided into three subgroups (1, 2 and 3). Animals were treated with the antibiotic twice daily for 7 and 10 days (subgroups 1 and 2, respectively) and samples were collected 12 h after the last treatment. In the subgroup (3), animals were treated with the same dose of antibiotic for 10 days and samples were taken 5 days after withdrawal of antibiotic (recovered group). Group III: For sperm abnormalities animals were treated with the same dose of antibiotic for 5 days and samples were collected at the days 7, 14 and 35 after the 1st oral administration (3 subgroups).

In all experiments, five animals were taken for each treatment, and the drug was administrated orally by using oral gavages.

2.6. Experimental procedure

2.6.1. Chromosomal aberration analysis in bone marrow and mouse spermatocytes

Mice were *i.p*. injected with colchicine 2.5 h before sacrificed. Bone marrow chromosomes were prepared according to the technique developed by Yosida and Amano [18] and modification by Fahmy *et al*. [19]. A hundred well spread metaphases were analyzed per mouse describing different kinds of abnormalities. Scoring was performed under 2500× magnification with a light microscope. Spermatocyte chromosomes were prepared from the testes of the same animals according to Evans *et al*. [20]. A total of 100 well spread diakinesis metaphase I cells were analyzed per animal scoring different types of abnormalities.

2.6.2. Histopathological study

After sacrificing the mice, parts of the liver, kidney, testis, brain and heart, tissues were collected for histological studies. The tissues were washed in normal saline and fixed immediately in 10% formalin for at least 24 h, dehydrated with alcohol, embedded in paraffin, cut into 4–5 μm thick sections, and

stained with hematoxylin–eosin dye for histopathological investigation [21]. Images were captured and processed using Adobe Photoshop Version 8.

2.6.3. Sperm shape abnormalities

Sperms were prepared according to the recommended method of Wyrobek and Bruce [22] and modification by Fahmy *et al.* [19] and smears were stained with 1% Eosin Y. A total of 1 000 sperms were counted per animal (5 000/each treatment), and different types of sperm abnormalities were scored (Head & Tail abnormalities). Sperm preparations were examined by light microscopy at 1 000× magnification.

2.7. Statistical analysis

The obtained data from control and experimental animals were subjected to analysis of variance (ANOVA, one way). Least significant differences were used to compare between means at probability 0.05.

3. Results

3.1. Cytogenetic study

The ability of amoxicillin + potassium clavulanate (AC) to induce the genotoxic effect in mouse bone marrow and spermatocytes was evaluated in Tables 1 and 2, respectively. Chromosomal aberration analysis was taken as a marker of genotoxicity in the present study. The results show that AC induced a moderate increase in both bone marrow and mouse spermatocytes. Such an effect was statistically non-significant. The results in Table 1 show that the percentage of the induced chromosomal aberrations in bone marrow-cells reached 5.80 ± 0.68, 6.20 ± 1.04 and 5.60 ± 0.90 at the three time intervals tested as compared with 3.40 ± 0.55 for control. On spermatocytes, these percentages reached 5.20 ± 0.50, 6.00 ± 0.40 and 5.40 ± 0.55 compared with 3.20 ± 0.45 for the control.

The results (Table 3) show that AC affects sperm morphology. It induced statistically significant percentage of sperm abnormalities (head and tail abnormalities). The percentage of abnormalities reached 8.10 ± 0.55, 9.86 ± 0.63 and 12.12 ± 0.58 at the three tested time intervals (7, 14 and 35 days after the 1st treatment, respectively). Different types of morphological sperm abnormalities in head and tail were detected. Head abnormalities were represented by changes in the shape (triangle, amorphous, banana, hookless) or in size (big head). Tail abnormalities were also observed after treatment with AC in the form of a coiled tail.

Table 2
Chromosomal abnormalities induced in mouse spermatocytes after treatment with amoxicillin–clavulanic acid.

Treatment time (d)	Period of harvest after last treatment	Total abnormal metaphases (*n*; %)	Types of metaphases (*n*)	
			XY-univalent	Autosomal univalent
Control	–	16 (3.20 ± 0.45)	10	6
7	12 h	26 (5.20 ± 0.50)	15	11
10	12 h	30 (6.00 ± 0.40)	20	10
10	5 d	27 (5.40 ± 0.55)	19	8

Total number of examined metaphases was 500 with 5 animals in each group; Total dose/day was 162 mg/kg b. wt.; Data are expressed as mean ± SE.

3.2. Histopathological examination

3.2.1. Liver

The histological examination of the liver sections in the control mice group showed a normal histological picture. The central vein lies at the center of the lobule surrounded by the hepatocytes with distinct nuclei and hepatic sinusoids (Figure 1A).

The sections of the liver in the group treated with antibiotic for 7 days showed mild congestion of central vein with moderate degeneration, necrosis and hydropic changes in hepatocytes. Liver hemorrhage and pyknotic nuclei were also noticed (Figure 1B).

The group treated for 10 days showed congestion of central vein, liver hemorrhage and severed hydropic changes in hepatocytes (Figure 1C). However, the 3rd group taken at the day 5 after treatment (10 days treatment) revealed the same lesion (Figure 1D).

3.2.2. Kidney

Histopathological examination of the kidney sections in the control group showed the glomerulus, urinary space, and Bowman's capsule, renal tubules (proximal convoluted tubules and distal convoluted tubules) (Figure 2A).

The sections of the kidney in the group treated for 7 days exhibited mild degenerative changes in glomerular and widening urinary space. Tubules degeneration, hydropic changes, and hemorrhage between tubules were also noticed (Figure 2B). Sever degenerative changes in glomerular and tubules in the form hyper cellularity in glomerular with reduced urinary space were noticed after 10 days treatment. Vacuolation, hemorrhage and cell infiltration between tubules with pyknotic nuclei were also detected (Figure 2C). The recovery group (5 days after the

Table 1
Chromosomal abnormalities induced in mouse bone marrow cells after treatment with amoxicillin–clavulanic acid.

Treatment time (d)	Period of harvest after last treatment	Total abnormal metaphases			No. of different types of metaphases			
		No.	Mean (%) ± SE		Gaps	Fragment. and/or break	Deletion	Polyploidy
			Including gaps	Excluding gaps				
Control	–	17	3.40 ± 0.55	2.20 ± 0.50	6	9	2	–
7	12 h	29	5.80 ± 0.68	3.80 ± 0.45	10	15	3	1
10	12 h	31	6.20 ± 1.04	4.20 ± 0.95	10	14	5	2
10	5 d	28	5.60 ± 0.90	3.20 ± 0.60	12	14	2	–

Total number of examined metaphases 500 (5 animals/group); Total dose/day = 162 mg/kg b. wt.

Table 3
Number and mean percentage of sperm abnormalities induced in mouse after treatment with amoxicillin–clavulanic acid.

Time of harvest after first treatment	Period of treatment (d)	Abnormal sperms		Types of sperm abnormalities (*n*)					
		No.	Mean ± SE (%)	Triangular	Banana shape	Amorphous	Without hook	Big head	Coiled tail
Control	–	139	2.78 ± 0.48	35	5	59	25	1	14
7	5	405	8.10 ± 0.55*	95	13	116	97	6	78
14	5	493	9.86 ± 0.63*	111	19	147	99	13	104
35	5	606	12.12 ± 0.58*	138	27	192	108	14	127

Total number of examined sperms 5000 (5 animals/group); Total dose/day = 162 mg/kg b. wt.; *$P < 0.05$ compared with control group.

last treatment) showed the same histopathological changes (Figure 2D).

3.2.3. Testis

Histopathological examination of control testis showed normal seminiferous tubules, and conventional arrangement of the spermatogenic cells, including spermatogonia, spermatocytes, and spermatids. Sertoli cells rested on the basement membrane. Leydig cells were a component of interstitial tissues (Figure 3A).

The sections of the testis in animals treated for 7 days showed a reduction in the number of spermatozoa, vacuolation, pyknotic cells and congestion of blood vessels (Figure 3B).

While the animals treated for 10 days showed atrophy of some seminiferous tubules, the basement membrane was interrupted. The spermatogenic cells of seminiferous tubules appeared disorganized, and spermatogenesis was reduced. Vacuolation, pyknotic cells and congestion of blood vessels were also noticed (Figure 3C). The recovery group revealed changes nearly to that of 10 days treatment (Figure 3D).

3.2.4. Brain

In control group, the cortical neurons are arranged in neat rows with abundant cytoplasm, and the nuclei are round and basophilic (Figure 4A).

After 7 days treatment, the structures of the cortical neurons showed minimal damage with pink shrunken neurons that are surrounded by perineuronal vacuolations (Figure 4B). Such a damage increased with prolonged treatment for 10 days (Figure 4C). However, the recovery group showed the similar appearance (Figure 4D).

3.2.5. Heart

The heart tissue of control mice showed regularly arranged myocardial fibers with centrally located nuclei of cardiomyocytes (Figure 5A).

The sections of heart in animals treated for 7 days showed minimal haemorrhages in the myocardium and pyknotic nuclei of cardiomyocytes (Figure 5B). Prolonged treatment and recovery showed moderate hemorrhages and mild disruption of

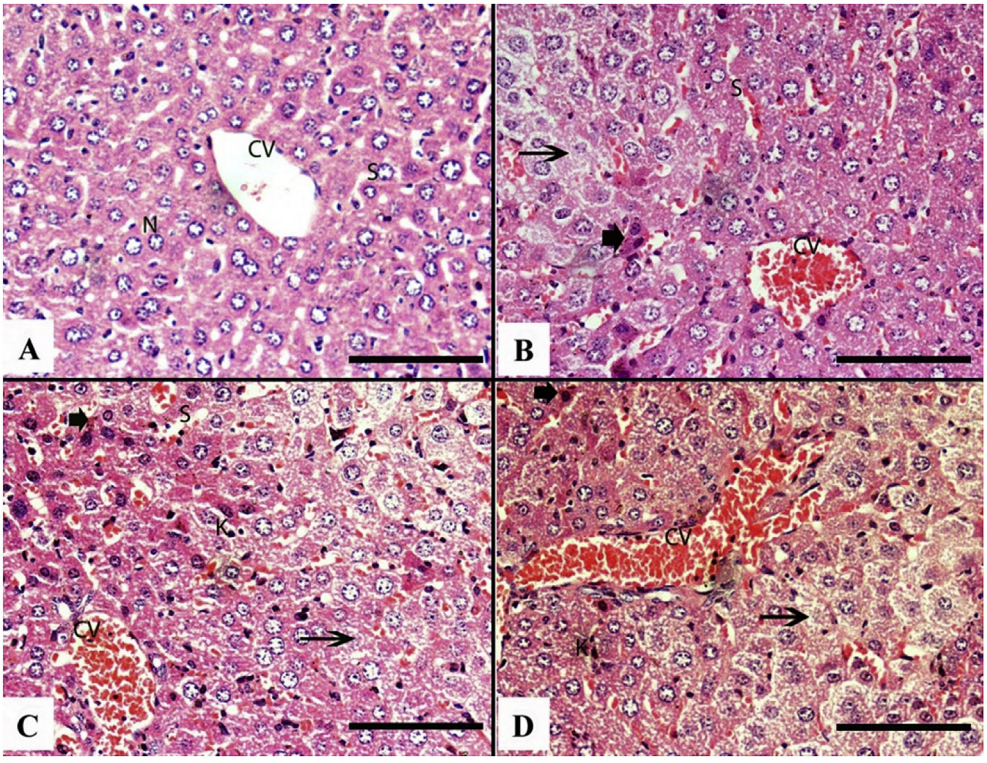

Figure 1. Photomicrograph of the liver sections of mice.
(A) Control liver showing normal central vein (CV), sinusoids (S) and nuclei (N); (B, C) Animals treated for 7 days and 10 days showing congestion of central vein (CV), degeneration in cells (arrow) and pyknotic nuclei (arrowhead); (D) Recovery group showing congestion of central vein (CV), degeneration in liver cells (arrow) and pyknotic nuclei (arrowhead) with activated Kupffer cells (K) (scale bar = 5 μm).

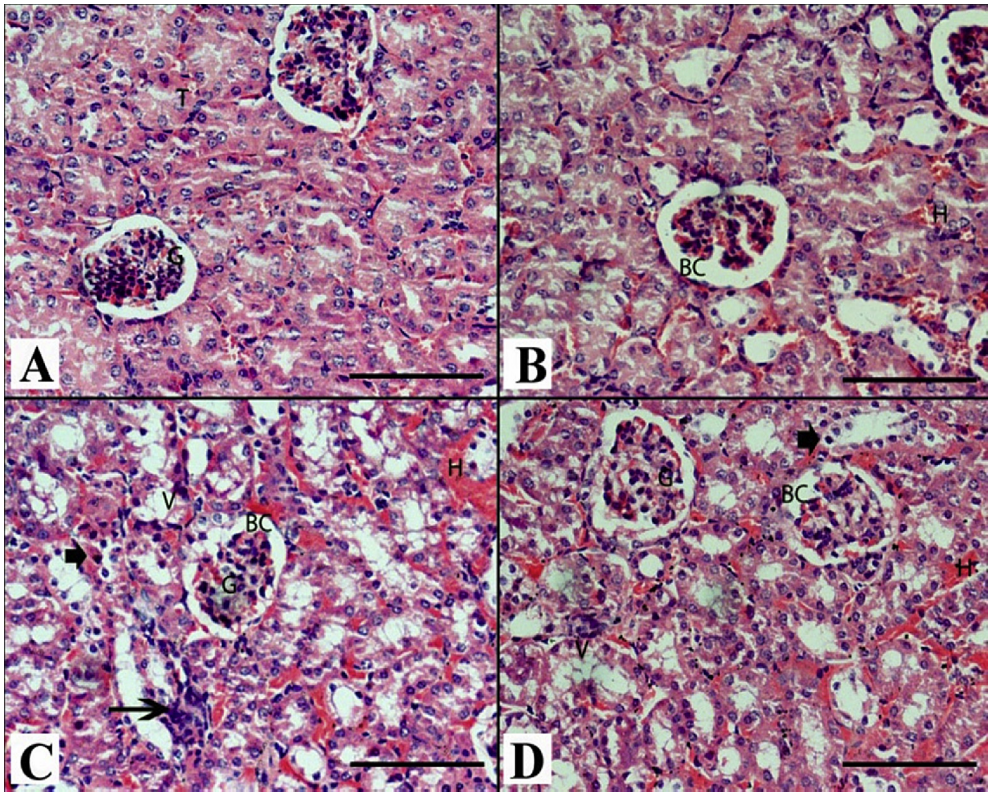

Figure 2. Photomicrograph of the kidney sections of mice.
For 7, 10 days treatment (B, C) and recovery group (D). (A) Control kidney showing normal glomeruli (G) and tubules (T); (B) Showing degenerative changes in glomerular and widening urinary space (BC) with hemorrhage between tubules; (C, D); Showing degenerative changes in glomerular (G), widening urinary space (BC). Tubules vacuolation (V), hemorrhage (H) and cell infiltration (arrow) between tubules with pyknotic nuclei (arrowhead) (scale bar = 5 μm).

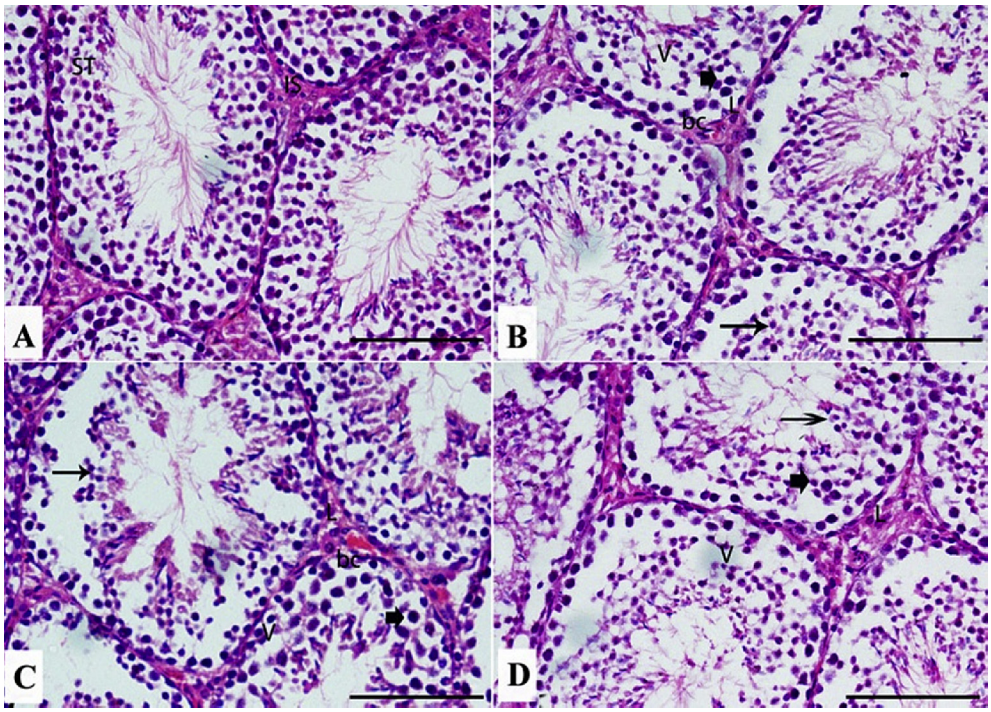

Figure 3. Photomicrograph of sections from testis.
Control (A) and treated mice (B, C, and D). (A) Control testis showing normal seminiferous tubules, spermatogenic cells (ST) and interstitium (IS); (B) Group treated 7 days showing mild degenerative changes in the spermatogenic cells, vacuolation (V), pyknotic cells (arrowhead), congestion of blood vessels (bc), degeneration of Leydig cells (L); (C, D): Showing sever degenerative changes in the spermatogenic cells, vacuolation (V), pyknotic cells (arrowhead), congestion of blood vessels (bc) degeneration of Leydig cells (L) and aggregate spermatozoa within the lumen (arrow) H&E (scale bar = 5 μm).

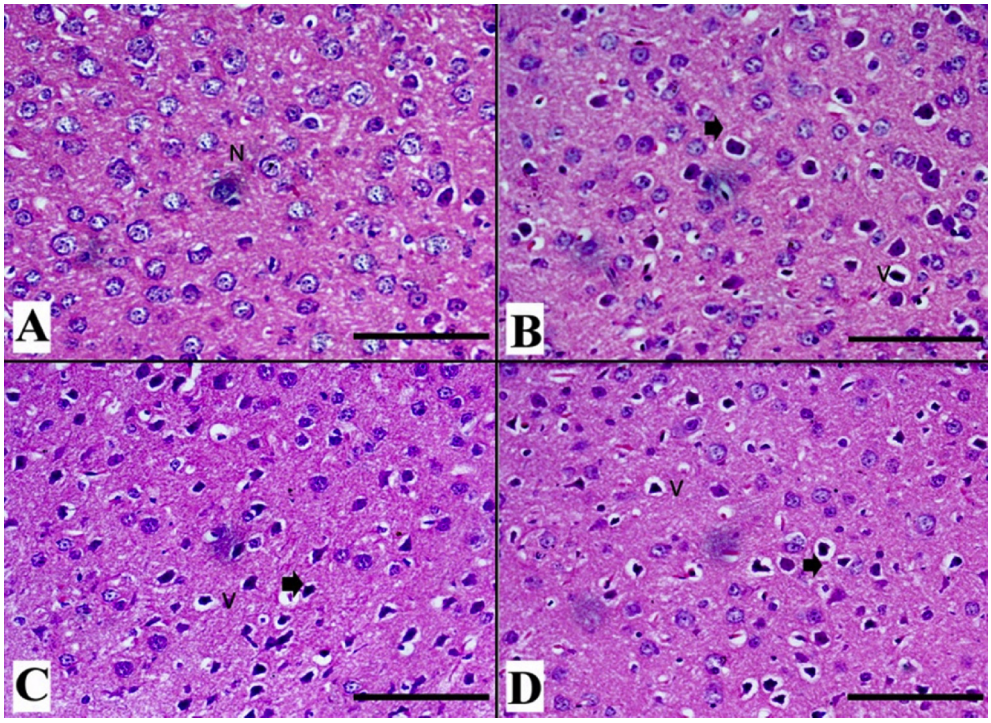

Figure 4. Photomicrograph of the cortical of brain sections.
Control (A) and treated mice for 7 and 10 days and recovery group (B, C and D) respectively: (A): Control brain showing neurons and nuclei (N); (B): showing minimal damaged with shrunken neurons (arrowhead) were surrounded by perineuronal vacuolations (V); (C, D): showing increased damaged with shrunken neurons (arrowhead) were surrounded by perineuronal vacuolations (V) (scale bar = 5 μm).

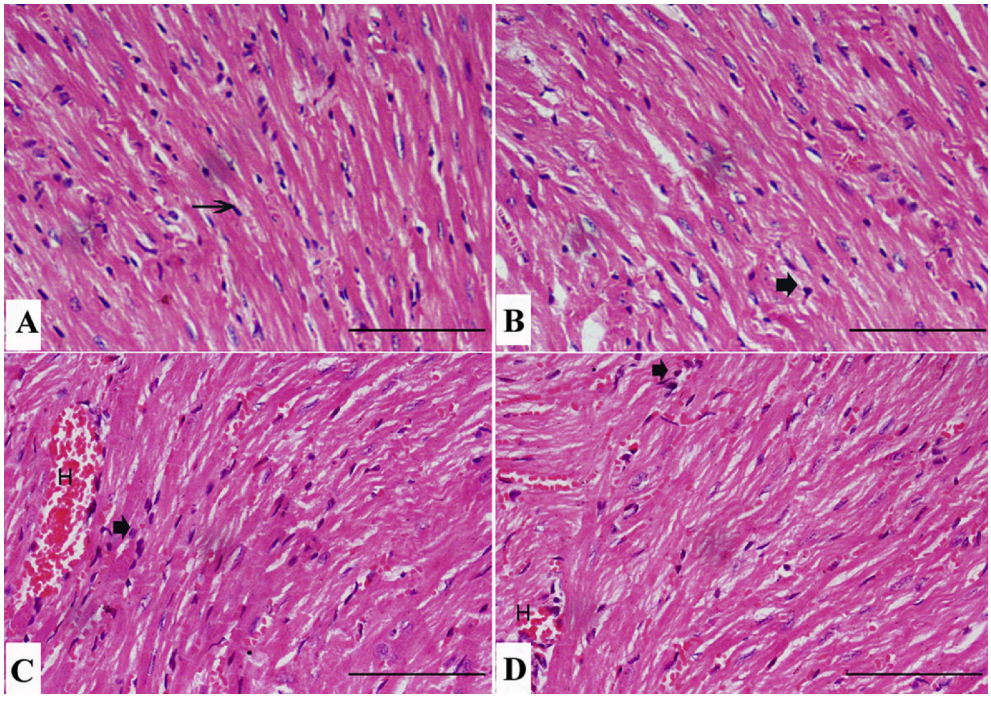

Figure 5. Photomicrograph of the heart sections.
Control (A) and treated mice (B, C and D). (A) Control heart showing regularly arranged myocardial fibers with centrally located nuclei of (arrow); (B) Group treated for 7 days showing minimal hemorrhages (H) in myocardium and pyknotic nuclei of (arrowhead); (C, D) Groups treated for 10 days and recovery showing moderate hemorrhages (H) and mild disruption of cardiac muscle fibers with pyknotic nuclei (arrowhead) (scale bar = 5 μm).

cardiac muscle fibers with pyknotic nuclei of cardiomyocytes (Figure 5C and D).

4. Discussion

Among all the pharmaceutical drugs that contaminate the environment, antibiotics occupy an important place due to high consumption rate in both veterinary and human medicine. Amoxicillin is on World Health Organization's (WHO) List of Essential Medicines. Amoxicillin–clavulanic acid, a drug commonly prescribed for the treatment of infections caused by susceptible bacteria, has been evaluated for its ability to induce genotoxicity evidenced by chromosomal aberrations in mouse bone marrow and spermatocytes. The results showed non-

genotoxic effect in both somatic and germ cells. Such results are in agreement with the findings of other authors [10] who investigated the potential genotoxicity of amoxicillin in peripheral blood lymphocytes *in vitro* using sister chromatid exchange (SCE), chromosomal aberration (CA) and micronucleus (MN) tests. The authors recorded no increase in the frequency of SCEs, CA or MN and suggested that amoxicillin does not pose a genotoxic risk to patients who are under therapy against bacterial infections. On the other hand, some authors reported that amoxicillin or similar antibiotics which are usually considered non-genotoxic have potential to injure genomic DNA possibly via the induction of intracellular reactive oxygen species [8,9]. Repair of the amoxicillin-induced DNA lesions was completed within several hours following the treatment of the drug [9]. These may explain the lower percentage of chromosomal aberrations which are recorded in the present study.

In the present investigation, AC has a pronounced effect on sperm. It induced a statistically significant proportion of morphological sperm abnormalities. The rate of abnormalities was proportionally associated with the time-intervals and reached approximately 2.9, 3.5 and 4.3 fold increases at the three time-intervals respectively as compared with the negative control. The results show that the maximum effect 12.12 ± 0.58 is at the day 35 after the first injection compared with 2.78 ± 0.48 for the control. Sperm cells observed at this time were presumably exposed to the drug while they were early primary spermatocytes and spermatogonia. While at 7 and 15 days (spermatozoa and early spermatid) the effect was less. This result indicated that different stages of spermatogenesis were affected after treatment with AC and there are remarkable differences in their sensitivity to the drug. AC induced both head and tail sperm abnormalities. Head abnormalities were represented by changes in the shape (amorphous, hookless, triangle, banana) or in size (big head). Also, tail abnormalities were observed after treatment with AC in the form of a coiled tail. The head abnormalities were reported to be a change in DNA content of sperm [23]. Moreover, Daris *et al.* [24] reported that head abnormalities especially amorphous heads are related to the high degree of DNA fragmentation. The tail is most probably responsible for sperm movement, so the distortion of tail or coiling may limit its movement and decrease fertility [19]. It was reported that non-motile sperm lacked the dynein arm which is an ultrastructural abnormality of microtubules in the tail motor complex [25]. There is evidence that many antibiotics affect sperm motility, count and morphology *e.g.* spiramycine [26], enrofloxacin [27], cefotaxime [28], Amoxicillin + clavulanic acid and ceftazidime [29], ampicillin in combination with sulphasalazine [30].

The results suggested that oral administration of Amoxicillin–clavulanic acid at the dose of 81 mg/kg b. wt. (twice daily) leads to disruption of spermatogenesis in the testes causing deterioration of motility and DNA content of sperm as well as morphological sperm abnormalities.

Sperm abnormalities detected in the present work could be due to oxidative stress pathway which generates ROS and lipid peroxidation as described by other authors after treatment with AC [9]. The sperm has high contents of polyunsaturated fatty acids in the plasma membrane, and thus they are highly sensitive to oxidative stress. The production of ROS, lipid peroxidation and the altered membrane can affect sperm leading to abnormalities [23]. Our results are also supported by the histopathological investigation of testis which was performed in the present study. A decrease in the number of spermatozoa, reduction of spermatogenesis and atrophy of seminiferous tubules was noticed after AC treatment.

Antibiotics are the largest group of agents that cause drug-induced liver injury (DILI). The present work showed that AC induced histopathological changes in liver cells. AC was previously reported to be one of the common causes of drug induced liver injury [31,32]. An association between AC-DILI and HLA alleles and the detection of drug specific T cells was recorded in patients with AC-DILI indicating that the adaptive immune system is involved in the disease pathogenesis [32]. Hepatotoxicity following AC has frequently been reported in adults but is rare in children. Its clinical presentation may vary, ranging from an asymptomatic elevation of liver enzymes to progressive cholestatic liver failure [33–35]. Nuclei irregularity, cytoplasm vacuolization, lymphocytic infiltration, destruction of bile duct epithelium, and ductopenia were detected after AC treatment [33]. This result is in agreement with the histopathological changes observed in the present study. The possible role of oxidative stress in hepatotoxicity induced by the drug has been previously established [12]. Additionally, Olayinka *et al.* [15] reported that two different combinations of amoxicillin/clavulanic acid (Augmentin® 375 and 625) induced renal and hepatic damage, altered enzymatic and non-enzymatic antioxidant defense system and induced oxidative stress in rats. These may explain the degenerative changes observed in the glomerular and tubules of the kidney tissue, and the sever hydropic changes in hepatocytes which was noted in the present study after AC treatment.

In the brain tissue, some damage were recorded in cortical neurons in the form of pink shrunken neurons surrounded by perineuronal vacuolations. Otli *et al.* [16] previously reported that Amoxicillin altered brain neurotransmitter levels in juvenile rats. It increased the glutamate levels in brain tissue significantly and decreased the activities of superoxide dismutase and catalase. The authors thought that the oxidative stress is the possible underlying mechanism of amoxicillin induced neurotoxicity.

Also, the histology of heart tissue showed that AC induced disruption of cardiac muscle fibers with pyknotic nuclei of cardiomyocytes.

In consideration of these facts, clinicians should be aware of reflecting carefully, whether the amoxicillin–clavulanic acid is necessary for treatment of patients with localized or uncomplicated infections. It is preferred to restrict the use of this combination in the treatment of infections with amoxicillin-resistant bacteria.

Conflict of interest statement

We declare that there is no conflict of interest.

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