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Assessment of Histopathological Changes in the Liver and Spleen of Mice Infected with *Salmonella typhi* Following Treatment with Ciprofloxacin

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Abstract

This study evaluated the toxicity of ciprofloxacin to spleen and liver when used for the treatment of mice infected with *S. typhi* for seven days. The dose concentration used in these experiments was 100mg/kg. Mice were divided into two groups . The first group (negative control) was not given ciprofloxacin, but rather a sterile phosphate buffer solution (PBS) as an alternative. Ciprofloxacin was administered to the second group. After seven days , the animals were sacrificed and organs (liver and spleen) were collected . The histopathological examination showed normal hepatocytes in the liver and normal structure of spleen cells in animals of control group . However, the treated group showed dilated and congested blood vessels with perivascular inflammatory cell cuffing and acute cell swelling in the liver, as well as white pulp activation with an increased number of megakaryocyte cells in the spleen. Therefore, the current study suggests that the concentration of 100 mg/kg of ciprofloxacin is considered to be toxic to hepatocytes and splenocytes of mice during the treatment period.

Keywords: Hepatotoxicity, spleen, mice; Ciprofloxacin, *Salmonella typhi*.

تقييم التغييرات النسجية في كبد وطحال الفئران المصابة ببكتيريا *Salmonella typhi* بعد معالجتها بالسبروفلوكساسين

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الخلاصة

قيمت هذه الدراسة سمية السيبروفلوكساسين الى الطحال ونشاط الكبد والمستخدم في علاج الفئران المصابة بكتيريا Salmonella. typhi ولمدة سبعة أيام. استخدم في هذه التجربة تركيز 100 ملغرام \ كيلو غرام. قسمت مجاميع الفئران إلى مجموعتين. المجموعة الاولى (السيطرة السالبة) لم تعامل بمضاد سيبروفلوكساسين ، ولكن استخدم المحلول الفسيولوجي الملحي المعقم كبديل, مضاد السبروفلوكساسين تم تجريعه الى المجموعة الثانية . بعد سبعة ايام تم التضحية بالحيوانات وجمعت الاعضاء(الكبد والطحال)لغرض فحصها نسجيا. اظهرالفحص النسجي لمجموعة الاولى (حيوانات السيطرة) لوحظ ان الشكل الطبيعي لخلايا الكبد كانت طبيعية وكذلك تركيب و خلايا الطحال ايضا سليما. بينما المجموعة المعاملة بالمضاد أظهرت توسع واحتقان بالأوعية الدموية مع تجمع الخلايا الالتهابية حول الاوعية الدموية وانتفاخ الحاد للخلايا في الكبد. نشاط في اللب الابيض مع تضخم بالخلايا المنتجة للصفائح الدموية في الطحال. أوحت الدراسة الحالية

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ان جرعة السيبروفلوكساسين 100ملغم \ كيلو غرام تعتبر ذات تأثير سمي على كبد وطحال الفئران اثناء فترة المعالجة.

Introduction

The use of antibiotics is considered as a general cause of DILI (drug-induced liver injury). Ciprofloxacin is a fluoroquinolones with a broad spectrum of anti-bacterial efficacy. However, ciprofloxacin has excellent bio-availability and safety following the oral administration [1]. Ciprofloxacin is active towards Gram positive and negative organisms. The mode of action of ciprofloxacin involves the blocking of the synthesis of bacterial DNA throughout inhibiting bacterial topoisomerase IV and DNA gyrase (topoisomerase II) [2]. Inhibiting topoisomerase IV interferes with the separation of the replicated chromosomal DNA in special daughter cells by cell division [3].The liver is considered to be the main organ that is responsible for the metabolism and excretion of chemotherapeutic agents, xenobiotics, and environmental pollutants; these substances, together with oxidative stress, are responsible for hepatotoxicity (liver injury) which is a main clinical concern [4]. Liver toxicity is related to cell necrosis, depletion of glutathione levels, and increased lipid peroxidation [5]. Salmonenella typhi is known to cause typhoid fever and, subsequently, liver injury. Delay in the diagnosis of infection, as well as the development of DILI, may result in acute liver failure and cirrhosis [6]. The spleen is a secondary lymphoid organ that is highly sensitive to various chemical materials. It consists of lymphoid and vascular elements and is the location of hematopoiesis. The spleen is also partially involved in the removal of the ageing and degenerating red blood cells, in addition to particulate materials and circulating bacteria from the blood circulation [7]. This organ is considered to be the location of indirect and direct toxicity, the location of malignant neoplasms arising in other locations, and the target for several carcinogens. The immune system undergoes continuous differentiation and self-renewal to maintain immunecompetence and , therefore, can be affected by xenobiotics that change the current cellular balance [7, 8].Materials and Methods

Bacterial strains and growth conditions

Salmonella typhi was isolated from human patients suffering from typhoid fever and then cultured in XLD and MacConkey agar. The isolate was incubated at 37°C for 18-24 hr [9]. The next day, prior to oral inoculation, it was confirmed that all animals were pathogen-free by using bacteriological examinations which showed that the mice had a negative culture for *S. typhi* . At the beginning of the study, the mice were deprived of food overnight and of water for 3 hrs. They were then fed with 0.2 ml of bacteria suspended in LB (Luria broth). The actual dosage was confirmed using the plating of serial dilutions of the inoculum, and prepared the LD50 dose of mic. The mice were gavaged with 0.2ml of *Salmonella typhi* suspension according to the LD50 dose, while the control group was gavaged with the same amount of sterile saline [10,11]. Colonies of bacteria were recovered from the intestines of mice. The mice were sacrificed and their intestines were extracted, homogenized in sterile phosphate-buffered saline (PBS) , plated on MacConkey and XLD agar plates, and incubated overnight at 37°C to obtain the results [10].

Estimation of Lethal Dose 50 (LD50)

The estimated viable count of the bacteria in each diluent was made according to the method of Miles and Misra [12].

Animal groups and ciprofloxacin doses

Twenty (Swiss albino) mice were used for the LD-50 experiment. Animals were aged between 6-8 weeks and weighed 25.4 ± 3.2 g. The animals were stabilized for one week before the commencement of the experiments. Commercial pelleted food and clean drinking water were provided. After stabilization, mice were randomly grouped into two groups of 10 mice each; group one was infected with *Salmonella typhi* . After 3 days, ciprofloxacin was followed for approximately 7 days. Group two was used as a control. Ciprofloxacin was administered orally, once per day , with a total daily dose of 100 mg \ kg of body weight, At 3 days post infection, a maxim-um dose was used according to previous methods [13,14]. The results were interpreted in relation to animal survival and observed toxicity [15].

Antibiotic solution preparation

Ciprofloxacin powder (Bioanalyse, Turkey) was dissolved in sterile distilled water and the MBC (minimum bactericidal concentration) and the MIC (minimum inhibitory concentration) were calculated according to an earlier work [16]. The solutions were transferred to sterile vials through

0.22 mm syringe filters. Ciprofloxacin was gavaged orally to mice with a concentration of 100mg/kg/day, [17].

Histological examination

Tissue specimens from the liver and spleen were collected for histological examination under the light microscope in accordance with standard protocols. The liver and spleen were removed from the mice used in the experiment. These organs were kept in 10% neutral buffered formalin (pH 7) for fixation . Fixation was achieved after 72 hrs and after that the organs were dehydrated by passing through ascending concentrations of ethyl alcohol–water, cleared in xylene and sequentially embedded in blocks of paraffin wax , which were then sectioned using a rotary microtome. Sections of tissues with three to five µm thickness were sliced, stained with H-E (Haemat-oxylin eosin), and then examined using a light microscope. The results of the histopathological examination were recorded using a digital camera system attached to the microscope [18- 20].

Results and discussion

1- Estimation of lethal dose (LD50) of *S.typhi*

In the present study , the lethal dose (LD50) of *S. typhi* in mice was found to be $3 \times 10^{8.49}$ cfu / ml, which is compatible with the previously published results [21- 23].

2- Evaluation of clinical signs

The medical assessment was performed depending on the clinical responses of each mouse to *Salmonella* infection. These included less efficacy of mice, appearance of fur, increased feeding, ataxia , hunched position, move abnormally(some time fast and jumps a lot other times), weight loss, tremor, and increased feces in the first week after infection. These responses may be due to infection with *Salm-onella* that causes diarrhea .The current results are in agreement with those previously published [10].

3- Antibacterial activity in mice

Mice from each group were treated with ciprofloxacin at 3 days post infection. Ciprofloxacin (100 mg/kg, 0.5 µg/ml) was given orally to mice infected with *S.typhi* for seven days. Histopathological examination of liver and spleen was then carried out [24].

4- Control group

In this study , liver and spleen specimens of the control (negative) group(without treatment) showed normal hepatocyte architectures and normal blood vessels (central vein) in the liver (Figure-1), as well as normal white and red spleen pulps (Figure-2). These results are in accordance with those of previous studies [25, 26] which include polygonal hepatocytes with granular eosinophilic cytoplasm and red blood cell (RBCs)-free central vein in the liver. The spleen showed a red pulp loaded with RBC and a white pulp that contains mixture of lymphocytes and plasma cells.

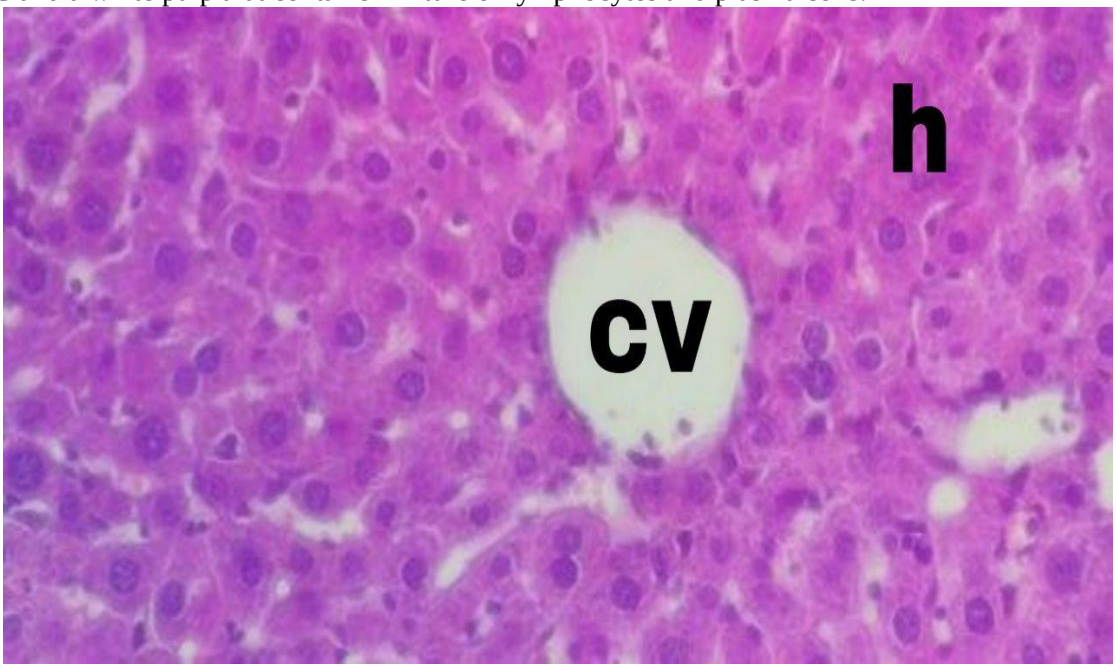

Figure 1-Liver section of the control group exhibiting normal architectures, including normal central vein (cv) and normal hepatocytes (h); stained with H &E, (40x).

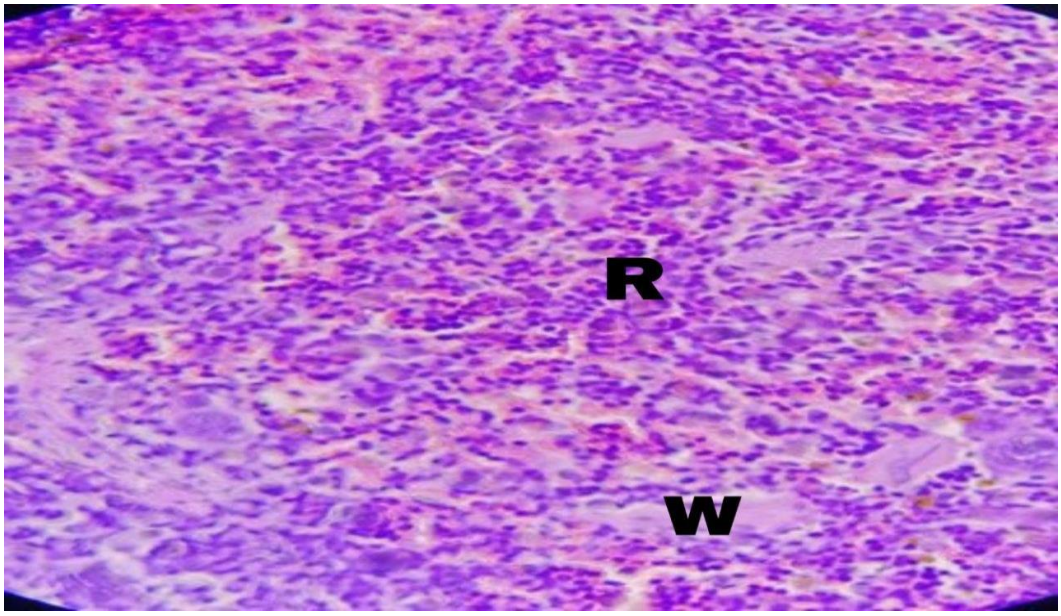

Figure 2-Spleen section of mice control group, exhibiting normal white (W) and red (R) pulp; stained with H&E, (40 x).

5- The effects of ciprofloxacin on the liver and spleen of *S.typhi* infected mice

The effects of ciprofloxacin on the liver and spleen of *S.typhi* infected mice were reflected by changes in the structure of these organs. In the liver, the results showed dilated and congested blood vessels with perivascular inflammatory cells and acute cell swelling (Figure-3).

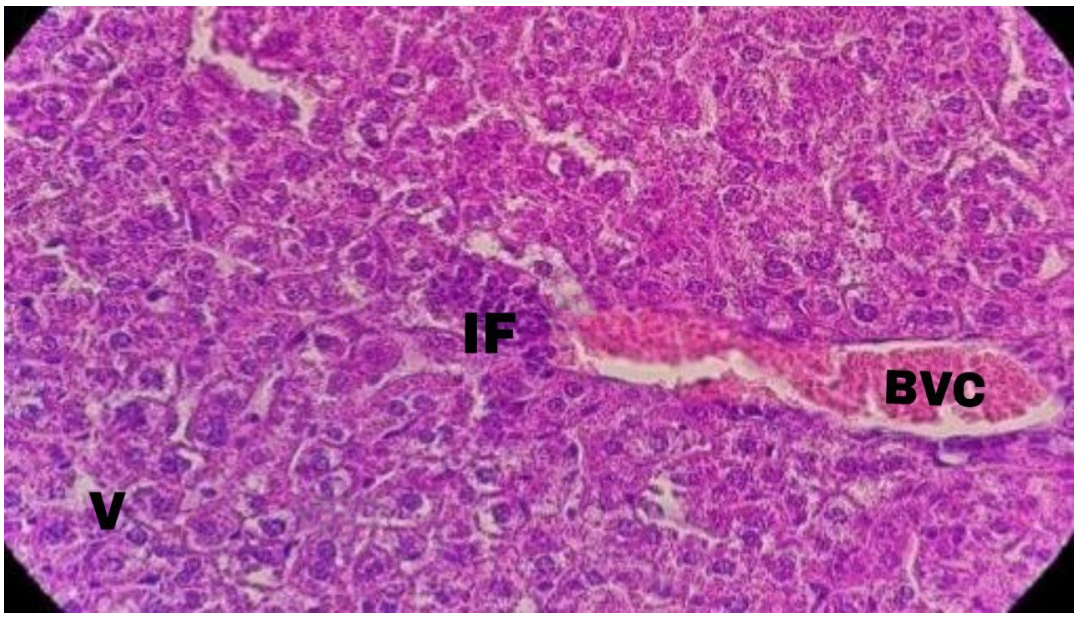

Figure 3-Liver section showing dilated and congested blood vessels, cytoplasmic vacuolation (V) , infiltration of inflammatory cell (IF) with perivascular inflammatory cell cuffing (BVC), and acute cell swelling ; stained with H&E, (40 x).

Acutely swollen hepatocytes could be attributed to mitochondrial injury, endothelial reticulum injury, and organ injury, which can lead to cell death, vascular damage, thrombosis, tissue/organ ischaemia, and necrosis [27]. The recorded hepatotoxic impacts of ciprofloxacin may be due to the

oxidative stress induced in the liver by the use of ciprofloxacin, through the generation of oxidative radicals leading to protein depletion in the hepatocyte. As a result, a decrease of nucleic acids and DNA damage can occur, leading to remarkable degeneration in mitochondria and reduction in the number of hepatocytes. This result is in agreement with those of previously published reports [28, 29].

The extending of the hepatic cells and the congestion of blood vessels in this study may be due to the hepatotoxicity of ciprofloxacin, which could be attributed to its active metabolites [30]. This result agrees with the findings of other studies [31, 32]. Ciprofloxacin was shown to inhibit eukaryotic and prokaryotic cell growth and protein synthesis by interfering with the replication of DNA and RNA. An earlier work [28] found a congestion of blood vessels and leukocytic infiltration in the liver of animals treated with ciprofloxacin. The pathogenesis mechanism of *Salmonella typhi* caused hepatic dysfunction, which was postulated to be either due to the endotoxemia with immune-assisted liver damage or through direct invasion [33]. Histopathological examination of liver tissue of animals infected with *S. typhi* showed the appearance of typhoid nodules , degeneration, swelling, and mononuclear cell filtratation [34]. In systemic salmonellosis, such as typhoid fever, Salmonella may target specific types of host cells, such as dendritic cells and macrophages that favor spread through the lymphatics and blood stream to the deeper tissues. This then leads to transport to the spleen, bone marrow, liver, and gall bladder and causes damages to cells [35]

Following ciprofloxacin treatment of *S.typhi* infected mice in the present study, the spleen tissues showed megakaryocyte hyperplasia and white pulp activation (Figure-4).

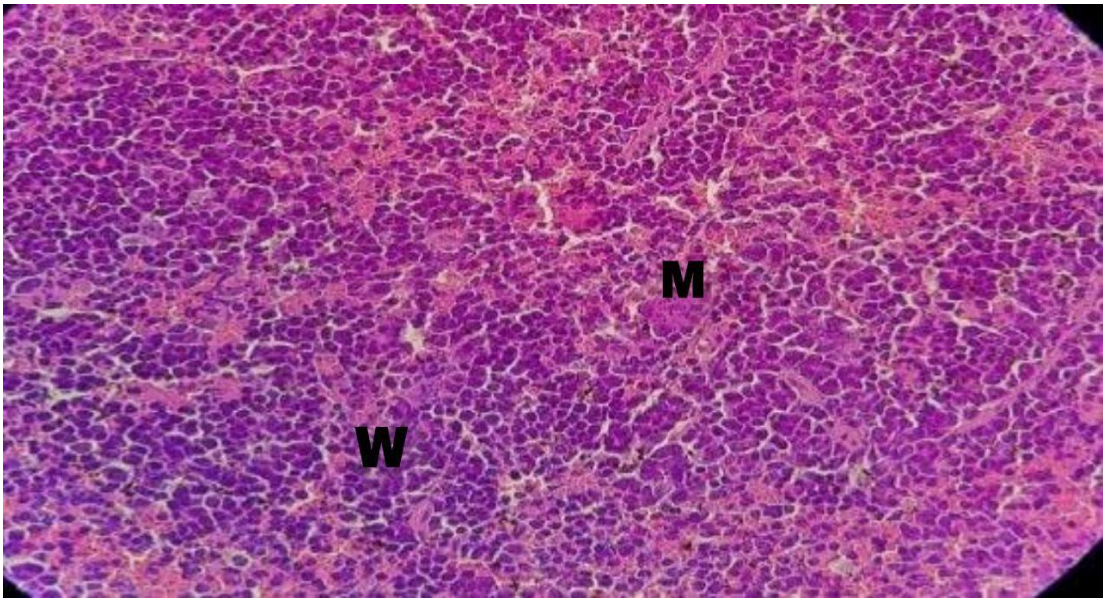

Figure 4-Section of spleen of mice infected with *S. typhi* and treated with Ciprofloxacin , exhibited white pulp activation(x), and megakaryocyte Hyperplasia (+),Stained with H&E, (40 x).

Treatment of mice with ciprofloxacin against *S. typhi* has been changed to extramedullary hematopoiesis in the spleen. Significantly, the megakaryocytes number was increased , which is in line with previously published findings [36,37]. The extra medullary hematopoiesis consisted of erythroid precursors, myeloid precursors, megakaryocytes (the precursors of platelets), or all the three components. The erythroid component, i.e. the erythroid hyperplasia, may lead to secondary hemorrhage or erythrocyte destruction, i.e. hemolytic or autoimmune anemia. Also, the myeloid component may predominate secondary to the inflammatory disorders, i.e. the myeloid or granulocyte hyperplasia. Many histological similarities with granulocytic leukemia were reported in myeloid hyperplasia [38], although some degree of extramedullary hematopoiesis occurred in mice. Extramedullary hematopoiesis was increased due to haematotoxic damage, systemic anemia, and bacterial infections that cause bleeding elsewhere in the body [7].

In particular, spleen was sensitive to enlargement due to the use of erythroid and myeloid hyperplasia, most of which were accompanied by megakaryocyte hyperplasia. The majority of spleens show lymphoid hyperplasia in white pulp in response to foreign antigens expressed by infectious

agents or tumors [39, 40]. Due to the use ciprofloxacin to treat mice infected with typhoid fever, bone marrow suppression and megakaryocytic hyperplasia were reported [41]. This result is in agreement with those of other studies [42, 43]. Ciprofloxacin is a very important antimicrobial against a wide range of aerobic bacteria species. This is due to the association between treatment with and histopathological changes. A previous study reported potent therapeutic effects of ciprofloxacin in the spleen along with the stimulation of the immune system[42]. The spleen is an organ that is present in almost every species of vertebrates. It acts primarily as a blood purifying filter of the destroyed bacteria and red blood cells. This organ is also an important organ in the immune system, producing white blood cells, and infections, and synthesizing antibodies [44- 46]. The active white pulp shows an increase in the number of lymphocytic cells, indicating an immune response, since splenic and hepatic macrophages are the main cells in which the bacteria reside (40).

References

1. Deepali S., Rahul P. P., Syed T R. Z., Md. Moklesur R. S., Qi Ying L., and Long C. M.(**2017).** Interplay of the Quality of Ciprofloxacin and Antibiotic Resistance in Developing Countries. *Front Pharmaco*l , **8**: 546. PMC5566961.
2. Alovero F. L., X. S. Pan., J. E. Morris, R. H. Manzo and L. M. Fisher. **2000** .“Engineering the Specificity of Antibacterial Fluoroquinolones, Benzene Sulfonamide Modifications at C-7 of Ciprofloxacin Change Its Primary Target in Strep-tococcus Pneumoniae from Topoisomerase IV to Gy-rase,” *Ant A and Chemoth*, **44**(2): 320-325. doi:10.1128/AAC.44.2.320-325.2000
3. Mitscher L. A. and Z. Mao. **2003.** “Structure Activity Relation-ships of Quinolones in Fluoroquin -olones Antibiotics,” In: A. R. Ronald and D. E. Low, Eds., *Birkhauser Based*, 2003, pp. 11-48.
4. Omodamiro, OD. **2018.** Hepatotoxicity Activity of Broad Spectrum Antibiotics (Amoxicillin and Ciprofloxacin) after Prolonged Administration in Wistar Albino Rats. SAJ Pharmacy and Pharmacology; **5**(2): 9-12.
5. Ostapowicz G, Lee WM **.2000**. Acute hepatic failure: a Western perspective. *J Gastroenterol Hepatol*, **15**: 480-8.
6. Prescott, LM**. 2002.** *Microbiology* (5th ed). The Mc-Graw Hill companies.
7. Andrew W. S. **2006.** Histopathology of the Spleen. *Toxicologic Pathology*, **34**: 466 –503
8. Vladimir N. ., Voloshin V. G., Koveshniko.v and Irina S. V. **2014.** Morphology of the spleen in adult albino rats after. *Int J Anat Res* 2014, **2**(2): 421-30. ISSN 2321- 4287.
9. Vandepitte. J., Verhaegen .J., Engbaek .K., Rohner. P., Piot. P and Heuck. C. C. **2003.** *Basic laboratory procedures in clinical bacteriology*, 2nd ed. World Health Organization Geneva. ISBN 92 4 154545 3.
10. Özkaya H., Abdullah B., Akcan, Gökhan A., Seçil A., Yasmin R., Gammon S.T and Jeff. M. **2012.** Salmonella *typhimurium infections* in balb/c mice: a comparison of tissue bioluminescence, tissue cultures and mice clinical scores. *New Microbiologica*, **35**: 53-59, 2012.
11. Boyle EC., Dombrowsky H., Sarau J., Braun J, Aepfelbacher M, Lautenschläger I, **. 2016.**Ex vivo perfusion of the isolated rat small intestine as a novel model of *Salmonella enteritis*. *A J Phy Gastro and Liver Phy*.; **310**(2): G55-63.
12. Miles, A.A. and Misra, S.S. **1938**. The estimation of the bactericidal power of blood . *J. Hyg*. **38**: 732-749.
13. Violeta R. C., Gema .d., Lorena H., Plínio N ., Vicente R., Ernesto G., Carmen P and Francisco S . **2010**. Comparative efficacy of novobiocin and amoxicillin in experimental sepsis caused by B-lactam-susceptible and highly resistant pneumococci. *Int J of Anti Agents*. doi:10.1016/j.ijantimicag.02.007.
14. Molly C. M., Shareef S., Lesley R., Matthew A. H., Ryan C., Lynn K. B.and Samuel L. M. A . **2018**. Evaluation of *in vitro* and *in vivo* antibiotic efficacy against a novel bioluminescent *Shigella flexneri*. Scientific Reports. **9**: 13567 | https://doi.org/10.1038/s41598-019-49729-2
15. Chairman.w.; Burrows.t.;cooper.s.; Jackson and Lawis. **2013**. invivo test method to identify the act toxicity estimate(ate) alternative method to the LD50 test .produced by working party of environment, Health and safety committe (EHSC)of Royal society of chemistry., (4): 1-5.
16. CLSI. **2019**. Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard, 29th ed., CLSI document M02-A11. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania19087, USA.

17. Machholz E., Mulder G., Ruiz C., Corning BF and Pritchett-C. KR .**2012**. anual restraint and common compound administration routes in mice and rats. *J. of Visu Exp*, **67**.
18. Luna, L.G. **1968.** *Manual of histologic staining methods of the Armed Forces Institute of Pathology*. 3rd Edition, McGraw-Hill, New York. **3**(5): 338-351
19. Bancroft JD. And Gamble M. **2013.***Theories and Practice of Histological Techniques*. 6th ed. New York, London and Madrid: Churchil Livingstone , **7**(12): 2768-2773.
20. Oluyinka. A. Iyiola., Temitope F. Olafimihan., Faoziyat A. Sulaiman and Abass T. Anifowoshe **.2018.**Genotoxicity and histopathological assessment of silver nanoparticles in Swiss albino mice *UNED Res J* of the costa Rican Ditance University (ISSN: 1659-441X) **10**(1): 102-109.
21. Benedict L.R. Non , M.D. and Flamiano R. M.S. **2004**. Use of bacteriophages as therapy for Escherichia coli-induced bacteremia in mouse models. *Infect Dis*. **33**(2): 47-51
22. Yousif AA, AL-Naqeeb MN. **2010**.Ultrastructural Changes in the Ileum of White BALB/C Mice Experimentally Infected with *Salmonella hadar*. *Am .J .of A and Vet Scie*.; **5**(3): 196-201.
23. Shallal Z. S. , Yousif A. A. and Al-Deresawi. **2016**. *Salmonella mbandaka* isolated from human: Clinical and gross pathological studies in experimentally infected mice. (Mirror of research in veterinary sciences and animals) *MRVSA*, **4**(1): 27-38.
24. Brigesh Shahare, Madhu Yashpal, and Gajendra Singh**. 2013.** Toxic effects of repeated oral exposure of silver nanoparticles on small intestine mucosa of mice. *Toxicol Mech Methods*,; **23**(3): 161–167.
25. Santosh P., Ansuman C., Shelley B., Manas, R. R**. 2010.** Histopathology and cell cycle alteration in the spleen of mice from low and high doses of sodium fluoride. *Res rep Flu*, **43**(4): 237–245.
26. Reem A. A., Wafa A. A., Dina M., Hind A., Nourah ., Ashraf M. B., Ahmed E. A. M., Tahani T. A and Manal E. **2019 .** Anti-Toxoplasma activity of silver nanoparticles green synthesized with Phoenix dactylifera and Ziziphus spinachristi extracts which inhibits inflammation through liver regulation of cytokines in Balb/c mice. *Biosc Rep*, 39 BSR20190379.
27. Minjun C., Ayako S.,Jürgen B., Raۛl J., Andrade M., Isabel L. **2015**.Drug-induced liver injury: Interactions between drug properties and host factors. *J of Hep*. **63**: 503–514.
28. Nadia H. I. **2006**. **"**Assessment of Histopathological and Histological Changes in Liver of Pregnant Female Rats Their Fetuses Following Ciprofloxacin Administration," *J of Egp Soc of Toxi*, **35**: 7-17.
29. Elias Adikwu. S, Nelson B. **2012**.Ciprofloxacin Cardiotoxicity and Hepatotoxicity in Humans and Animals. *J of Pharm & Pharmacy*, **3**: 207-213.
30. Higuchi K., E. Umegaki, T. Watanabe, Y .Yoda, E Morita, M. Murano, S .Tokioka, T .Arakawa .**2009**. Present status and strategy of NSAIDs-induced small, bowel injury. *J of Gastro*; **44**: 879-888.
31. Minuk G., Assy N. and Ding .L. **1997**. "Effects of Quinolone Antibiotic on Hepatic Growth and Protein Synthesis Fol-lowing Partial Hepatectomy in Rats," *J of Gastroenterol and Hep*, **12**(1): 5-7.
32. Channa, M.A. and Janjua, M.Z**. 2003.** Effects of ciprofloxacin on foetal hepatocytes. *J. Pak. Med*. Assoc. **53**: 448e450.
33. Morgatern. R and Hayes .PC. **1991**. The liver in typhoid fever: Always affected, not just a complication. *Am J Gastroenterol*; **86**: 1235-9.
34. Khosla .SN., Singh R., Singh .GP and Trehan VK. **1988**. The spectrum of hepatic injury in enteric fever. *Am J Gastroentrerol* ; **83**: 413-6.
35. Thomas R., Laura Mc., Smita G. and Denise M . **2012** . Salmonella's long-term relationship with its host. *FEMS Microbiol Rev*, **36**: 600–615
36. Dkhil, M. **2009.** Apoptotic changes induced in mice splenic tissue due to malaria infection. *J. Microbiol. Immunol. Infect*. **42**: 13–18.
37. Sun S, Wang W, Latchman Y, Gao D, Aronow B, Reems JA. **2013**. Expression of plasma membrane receptor genes during megakaryocyte development. *Physiol Genomics*. **45**(6): 217-227.
38. Long, R. E., Knutsen, G., and Robinson, M. **1986.** Myeloid hyperplasia in the SENCAR mouse: differentiation from granulocytic leukemia. *Environ Health Perspect*, **68**: 117–123.

39. Leung KY, Finlay BB. **1991**. Intracellular replication is essential for the virulence of Salmonella typhimurium. *Proc Natl Acad Sci USA*; **88**: 11470–11474
40. Ward JM., Rehg JE and Morse HC III. **2012.** Differentiation of rodent immune and hematopoietic system reactive lesions from neoplasias. *Toxicol Pathol,* **40**: 425-434.
41. Dutta. TK and Badhe .BA**. 1999.** Ciprofloxacin-induced bone marrow depression . *Postgrad Med J.* Sep; **75**(887): 571-3.
42. Risaku F., Lynnette H. C., Nikolai V. G., Eric D. L., Thomas B. E., Juliann G. K. **2013**.Ciprofloxacin Modulates Cytokine/ Chemokine Profile in Serum, Improves Bone Marrow Repopulation, and Limits Apoptosis and Autophagy in Ileum after Whole Body Ionizing Irradiation Combined with Skin-Wound Trauma. PLOS ONE | www.plosone.org | 8 : e58389.
43. Hua-Hsing L., Fei-Peng C., Rong-Kai L., Chun-Lin L and Ko-Tung. C **.(2015).** Ginsenoside Rg1 improves bone marrow haematopoietic activity via extramedullary haematopoiesis of the spleen. *J. Cell. Mol. Med.* **19**(11): 2575-2586**.**
44. Mebius, RE; Kraal, G. **2005**. "Structure and function of the spleen". *Nat Rev. Imm.* **5** (8): 606–16.
45. Firoz M. M., Elisabeth A P., Meghan J C and Ali A .A **.2011** . Humanized mice are susceptible to Salmonella typhi infection. *Cell & Mol Imm,* **8**: 83–87.
46. Elida M., Irena KP., Nevena K .**2015**. changes of spleen in wistar rats exposed to therapeutic doses of dexamethasone and medroxyprogesterone acetate evaluated by stereological parameters . contributions. *Sec. of Med. Sci*., XXXVI 3.