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IN VIVO ASSESSMENT OF ROSUVASTATIN EFFICACY ON EXPERIMENTAL MURINE WITH AVIRULENT TOXOPLASMOSIS

By

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Abstract

Toxoplasmosis is a contagious illness that is brought on by the parasite *Toxoplasma gondii*. The strain of *Toxoplasma gondii* may have an effect on how severe the symptoms of toxoplasmosis are. Immunocompromised people are more likely to develop neurological, ocular, and systemic diseases as a result of toxoplasmosis. Rosuvastatin® is one of the promising drugs in the treatment of toxoplasmosis. This study determined the rosuvastatin efficacy in treating murine toxoplasmosis. This is being done in an effort to make up for the deficiencies that are present in other standard medications. Mice were divided into 7 groups of 10 mice each. GI: Neither infected nor treated (normal control). GII: Infected, non-treated mice (positive control). GIII: Mice given Spiramycin® as prophylaxis before infection. GIV: Mice given rosuvastatin as prophylaxis before infection. GV: Mice infected and treated by spiramycin after 6th week for 2 weeks. GVI: Mice infected and treated by rosuvastatin after 6th week for 2 weeks. GVII: Following the sixth week, infection was confirmed, and treatment consisted of a combination of rosuvastatin and spiramycin for two weeks. Histological examination of brain and liver, and counting brain cysts, were used to evaluate the efficacy of treatment.

The results showed that rosuvastatin treated group showed significant reduction in brain cysts and improved histopathological picture in brain and liver tissue. Combination of rosuvastatin and spiramycin gave the best results in reduction of brain cysts number and the least pathological changes in brain and liver tissue. The effect of rosuvastatin was augmented after combination with spiramycin.

Keywords: Toxoplasmosis, Statins, Rosuvastatin, Spiramycin, Mice.

Introduction

It was estimated that one-third of the world's population is infected by *Toxoplasma gondii* (Duffy *et al*, 2019). Infection is asymptomatic in the majority of immunocompetent patients (Saleh *et al*, 2014), but immunocompromised patients can experience severe neurologic, ocular, pulmonary and disseminated diseases (Morsy *et al*, 2002). Cats are the definitive hosts pass millions of oocysts (sporozoites) in their feces (Al-Kappany *et al*, 2010), which spread in the environment contaminating the soil, water, fruits and vegetables in turn infect man, animals and birds (Dubey, 2009). The effective toxoplasmosis treatment is a combination of pyrimethamine and sulfadiazine and folic acid, which work together to inhibit *Toxoplasma gondii* folic acid production (Al-Ag- roudi *et al*, 2014). Spiramycin is a relatively safe macrolide antibiotic that inhibits protein synthesis in *Toxoplasma gondii*. Acute toxoplasmic pregnant woman didn't develop fetal infection, to prevent transfer of *T. gondii* to her baby (Petersen 2007). This medication, however, has several negative side effects, including hypersensitivity, bone marrow suppression, and teratogenic consequences (Hattoufi *et al*, 2022).

Rosuvastatin is a kind of statin drug used to reduce one's cholesterol levels, homozygous familial hypercholesterolemia, hyperlipidemia, mixed dyslipidemia, primary dysbetalipoproteinemia, hypertriglyceridemia, and prevention of cardiovascular disease as approved by the FDA (Bajaj *et al*, 2021).

It is hypothesized that rosuvastatin may have an effect on critical targets that are ac-

countable for the continued existence of T. gondii. The use of rosuvastatin in vitro was shown to be effective in preventing the parasite from reproducing and in dampening the inflammatory response brought on by the infection (Gouvea *et al,* 2013).

The efficacy of rosuvastatin in treatment of toxoplasmosis and when contrasted with the standard toxoplasmosis treatment, which consisted of a combination of pyrimethamine, sulfadiazine and folic acid (Abdel Malek *et al,* 2018), it demonstrated a considerable decrease in both the number of infected cells and the growth index of the internal *T. gondii* parasite (Nishi *et al,* 2020).

The present study aimed to evaluate Rosuvastatin® in treating murine toxoplasmosis. This was done in an effort to circumvent the adverse effects that are associated with the use of more conventional medications (Sciacchitano *et al*, 2018).

Materials and Methods

This study was done in Department Parasitology, Faculty of Medicine, Benha University and Department of Zoonotic Diseases, Theodor Bilharz Research Institute (TBRI). The protocol was approved by Research Ethics Committee, Faculty of Medicine, Benha University, which went with the Ethical Guidelines of Helsinki (6th Revision, 2008). The study started from August 2021 to Octo-ber 2021.

Parasite and animals: A total of 70 clean laboratory bred Swiss Albino male mice aged about 10 weeks and weighed 20-25gm were used. The non-virulent ME49 strain of *T. gondii* was kindly provided by the zoonosis section at TBRI. In order to establish chronic toxoplasmosis, the strain was routinely maintained through the process of repeatedly inoculating male Swiss albino mice with 0.1 milliliters of brain homogenate from previously infected mice containing approximately $1x10^2$ tissue cysts per milliliter for a period of six weeks. The animals were given a commercial complete food combination to eat and water from the tap to drink, and they were kept in a regulated environment with regard to temperature and lighting; 12 hours of light and 12 hours of dark cycle at laboratory temperature of 25±2ºC (Djurković-Djaković *et al,* 2002). Mice were divided into GI: Non-infected, non- treated (normal control). GII: Infected, non-treated mice (infected control). GIII: non infected mice given spiramycin® as prophylaxis before infection. GIV: non-infected mice given rosuvastatin® as prophylaxis before infection. GV: Infected mice and treated by spiramycin after 6th week for 2 weeks. GVI: infected and treated orally by rosuvastatin after 6th week for 2 weeks. GVII: Following sixth week, infection was treated using a mixture of rosuvastatin and spiramycin for two weeks as well.

Drugs: Rosuvastatin® (Crestor 40), a subclass of statins, manufactured by Astra-Zeneca, in tablets form, which were broken up, dissolved in distilled water and given in a dose of 40mg/kg/day (Nishi *al,* 2020). Spiramycin® (Spirex) as 3 M.I.U (1gm) manufactured by Medical Union Pharmaceuticals Egypt. Drug provided in tablets form, which were broken up, dissolved in distilled water and given in a dose of 200mg/kg/day (Djurković-Djakovi*ćet al,* 2002). So, the calculated dose for each mouse was 5 mg per day taken via tube feeding.

Experimental Design: Intraperitoneal injections of 0.1 milliliters of a brain cysts solution contained 10 cysts were inoculated each mouse.

Drug efficacy: Counting brain cysts by using a sonicator to mince one half of each brain separately for each mouse, 10% formalin was added to the minced brain tissue of each mouse in order to create brain emulsion homogenate and 0.1ml was applied and spread on the glass slide to dry at the room temperature. Slides were fixed with methanol acetone free and stained in Giemsa stain (1:10) for 45 minutes. The films were then washed, dried, and examined using light microscope at x40 or x100 if indicated after adding oil. Each slide was divided into 5 fields and number of cysts in each field was calculated as the mean cysts number.

Histopathological assessment of brain and liver tissue: After collecting tissue samples from brain and liver of each group, samples were s fixed in 10% neutral buffered formalin and processed for paraffin sectioning (5μm), stained with hematoxylin and eosin (H&E), and microscopically examined for pathological changes.

Statistical analysis: Data were collected, computerized and analyzed using Statistical Package for Social Science (SPSS) for Windows version 10. Data were shown using the mean together with the standard deviation (SD) (number of samples: ten), analyzed by Student t, and/or ANOVA for significant differences between groups. Post hoc test, Bonferroni for pair-wise determined the degree of difference between each pair of groups. P was less than 0.05 indicated significant differences. Formula used to determine reduction rate was: (Mean value of infected untreated group - Mean value of infected treated group) X 100 / Mean value of infected untreated group.

Results

Brain treated with spiramycin and rosuvastatin showed highest significant decrease in cyst count to 71.53%. Mice treated by spiramycin 2 weeks after infection showed 53.89% reduction, but with rosuvastatin 2 weeks after infection showed 35.92% reduction. Prophylactic mice treated by spiramycin 2 weeks before infection showed 27.81% reduction.

Histopathological brain tissue in positive control (GII), showed neurophagia of degenerated neurons with marked necrosis of neurons, appearance of neurofibrillary tangles and multiple large cysts scattered throughout parenchyma. Marked vasculitis and perivasculitis and multi-ple focal necrosis associated with glia cells infiltration and perivascular cuffing with mononu-clear inflammatory cells. But treated groups with rosuvastatin (GIV) and therapeutic (GVI) showed reduced in pathological changes especially in combined rosuvastatin and spiramycin (GVII) with minimal neurophagia and interstitial edema compared to other treated groups and blood vessels without lymphocytic infiltration. Liver tissue in (GII) showed portal tract infi-ltration and interstitial edema and marked hydropic degeneration and sinusoidal infiltration by mono and polymorh nuclear inflammatory cells and disturbed hepatic lobular architecture. But treatment with rosuvastatin (GIV) and therapeutic (GVI)) showed reduced pathological changes especially with combined rosuvastatin and spiramycin (GVII), which didn't show pa-thological changes except minimal hydropic degeneration of hepatocytes, but with neither sinusoids congestion nor lymphocytic infiltration or lobular necrosis. Details were given in table (1) and figures (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 & 17).

Table 1: Comparison of *T. gondii* cyst number recovered from brain among groups (n= 10).

Groups	Dose day/mg/kg	*T. gondii* cysts	Reduction %	P- value
GI: Normal control	_	0±0	-	-
GII: Infected control	_	1429.5 ±69.3[a]	-	--
GIII: Prophylaxis spiramycin	200	1032.0 ±41.2[c]	27.81	<0.001**
GIV: Prophylaxis rosuvastatin	40	1152.0 ±72.8[b]	19.41	<0.001**
GV TTT spiramycin	200	659.0 ±40.4[e]	53.89	<0.001**
GVI: TTT rosuvastatin	40	916.0 ±38.1[d]	35.92	<0.001**
GVII: TTT both (R+S)	R 40 S 200	407.0 ±29.8[f]	71.53	<0.001**

*P<0.05: Significant, ** P<0.001: Highly significant, # P>0.05: non-significant abc superscript letters means

Discussion

Generally, the steroids and isoprenoids are essential lipids for *T. gondii* metabolism and interference in the initial protozoan isoprenoid biosynthesis processes has been proposed as a mechanism involved in antiparasitic effects of statins. Statins also have anti-inflammatory properties, anti-blood clotting effects, and plaque-stabilizing properties. (Li *et al*, 2013).

In the present study, the mean number of cysts in the brain was significantly decreased in the prophylactic group treated by spiramycin 2 weeks before infection showed a reduction of 27.81%. Meanwhile, the prophylactic mice treated by rosuvastatin 2 weeks before infection showed significantly decreased in mean number of *T. gondii* cysts in brain with a reduction of 19.41%. But, combination of both prophylactic drugs showed more reduction in the brain cysts.

In the present study, the mean number of cysts in brain was significantly decreased in mice treated by spiramycin 2 weeks after infection showed a reduction of 53.89%. Meanwhile, the mice treated by rosuvastatin 2 weeks after infection showed a significant decrease in mean number of cysts in brain with a reduction of 35.92%. Consequently, spiramycin markedly decreased the number of brain cysts. This agreed with Grujić *et al.* (2005) who reported that a 3-week course of spiramycin 100mg/kg/day and a 4-week course of 200mg/kg/day in mice infected by cysts of *Toxoplasma* Me 49 strain, significantly enhanced protection, with marked reduction of brain cyst burdens at 6 months post infection.

In the present study, rosuvastatin showed effectiveness in toxoplasmosis treatment in vitro, it showed a significant reduction in both number of infected cells and proliferation index of the intracellular parasite as compared to the conventional treatment of pyrimethamine, sulfadiazine and folic acid (Sanfelice *et al*, 2017). Also, there were reduced levels of cytokines IL-6 & IL-17 by the anti-proliferative activity (Sanfelice *et al*, 2019).

Evangelista *et al.* (2021) reported that rosuvastatin reduced cyst load in brain of mice infected with *Toxoplasma* ME-49 strain, attenuated signs of brain inflammation such as inflammatory cell infiltration and tissue damage

In the present study, the mice treated with combination of rosuvastatin and spiramycin for 2 weeks after *T. gondii* infection showed the best reduction in mean number of cysts in the brain with a reduction of 71.53%. This also agreed with Nishi *et al.* (2020) who reported that rosuvastatin treated toxoplasmosis infected mice showed reduction the cysts number in brain tissue. They added that the infected control ones suffered from complication as such meningitis, inflammatory cell infiltration and perivascular cuff of 71 days post infection. This also agreed with the present findings of persistent inflammatory patterns in chronic toxoplasmosis phase. Moreover, Evangelista *et al.* (2021) evaluated rosuvastatin efficacy in vivo on toxoplasmosis chronic infection (ME-49 strain) by using 2 doses of rosuvastatin 10mg/kg/day and 40 gm/kg/day and reported that the brain parasite load was significantly lower in infected mice treated with rosuvastatin 40mg/kg/day compared to positive control ($P < 0.05$).

In the present study, the histopathological changes were detected in mice brains as well as inflammatory conditions; including necrosis areas in infected control ones. This indicated that active infection caused marked pathological tissue injury. This inflammatory condition was attenuated in rosuvastatin treated mice, especially with a dose 40mg/kg/day. This agreed with Cho *et al.* (2019) who reported that rosuvastatin markedly reduced cerebral parasitic load and interfered with the chronic toxoplasmosis. Previously, Sanfelice *et al.* (2017) reported that the activity of rosuvastatin in HeLa cells infected with *T. gondii* caused a significant reduction in number of infected cells and the proliferation index of the intracellular parasite as compared with the conventional toxoplasmosis treatment (pyrimethamine, sulfadiazine and folic acid). Nevertheless, the brain pathological alterations were attenuated in the rosuvastatin treated mice, which showed more intense glial cell proliferation and gliosis area, indicating tissue repair.

Other studies reported that the *T. gondii* ME49-infected mice exhibited sustained cerebral inflammation even during chronic phase of disease and showed that rosuvastatin

reduced the brain inflammation and cerebral parasitic load in the chronic phase of infection with the same dose (40mg/kg/day) used in the present study (Nishi *et al*, 2020).

In the present study, histopathological examination of mice brain was used as another evaluating parameter to study the efficacy of rosuvastatin. The present study positive control mice showed marked and sever neurons necrosis and neuronophagia with appearance of neurofibrillary tangles and multiple large cysts scattered throughout brain parenchyma. Also, there was marked vasculitis and perivasculitis as well as multiple focal necrosis associated with the glia cells infiltrated and perivascular cuffing with mononuclear inflammatory cells. This agreed with Dumas *et al.* (1994) who recorded that the histological examination of positive control mice showed inflammatory infiltrates and encephalitis and the brain lesions were characterized by a perivascular leucocyte infiltration (mainly around intracerebral and meningeal blood vessels), presence of microglial nodules and necrotic foci. Also, this agreed with Mahmoud (2007) who found that *Toxoplasma*-infected brain lesions showed perivascular cellular infiltrate, microglial nodules and necrotic foci.

In the present study, liver tissue of ME49 strain infected mice showed reduction in inflammatory infiltrate with regeneration of hepatocytes together with minimal mononuclear inflammatory infiltrate. Best result was obtained in mice treated with both spiramycin and rosuvastatin in minimal degree of hydropic degeneration, portal and sinusoidal infiltration and normal hepatic lobular architecture followed by spiramycin treated ones, and then rosuvastatin treated mice. The rosuvastatin improved endothelial function by increasing endothelial nitric oxide and reducing oxygen derived free radicals, which in turn reduced liver endothelial dysfunction and reduced high sensitivity C reactive protein inhibiting platelet aggregation to leukocytes and stopped formation of clots in injured endothelium (Bradbury *et al*, 2018). Also, spiramycin gave marked effect on both survival time and parasite load in liver, spleen, and brain by parasite protein synthesis inhibition (Farahat *et al*, 2020).

Conclusion

The outcome data showed that Rosuvastatin® gave significant effect against *T. gondii* and its efficacy increased when combination with Spiramycin® with marked decrease in the brain cysts count with the pathological improvement of liver and brain.

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Explanation of figures

Fig. 1: Mean values of *T. gondii* brain cyst count among groups

Fig. 2: Mouse brain from control (G I) showed normal histological structure (H&E, X400).

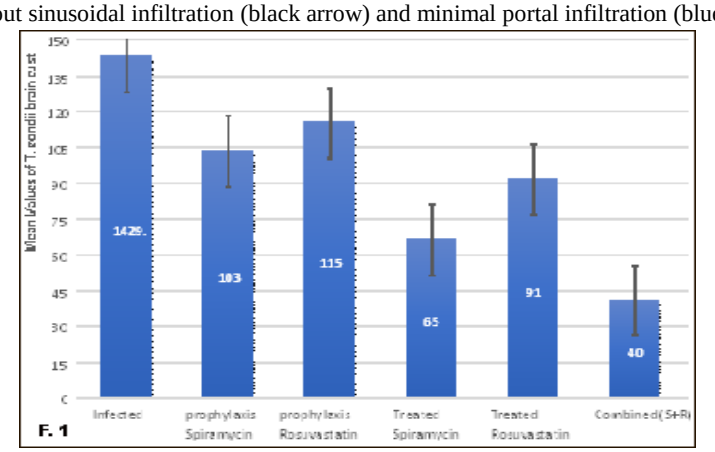
Fig. 3: Mouse brain from positive control (GII) showed meningitis, with aggregations of macroglial cells (orange arrow) (H&E, X400).

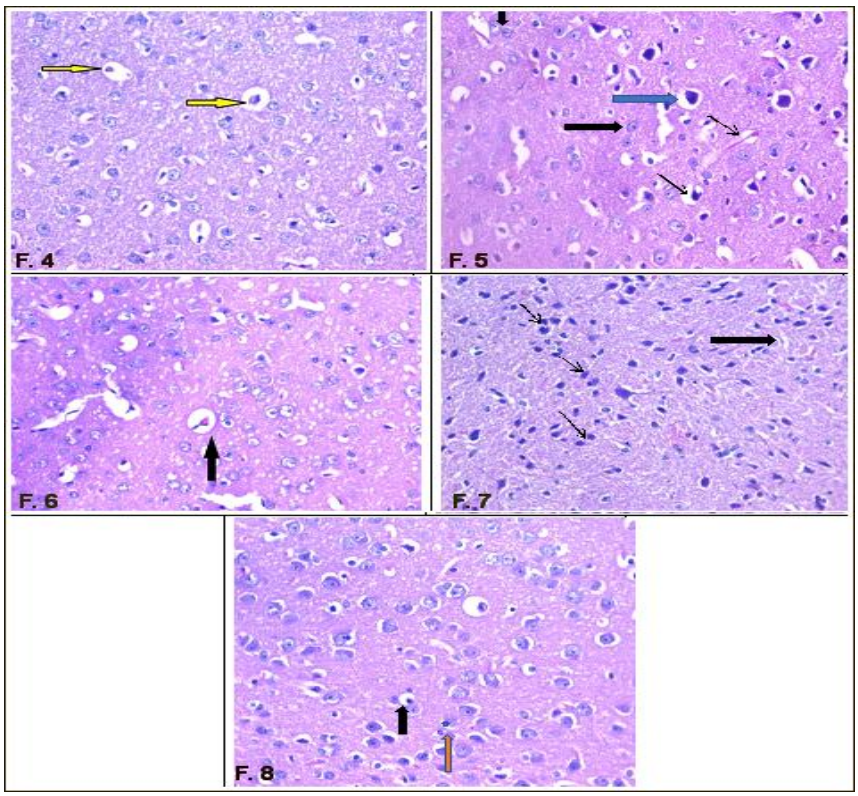
Fig. 4: Mouse brain treated with spiramycin (GIII) showed mild necrosis of neurons and neuronophagia (yellow arrow) (H&E, X400).

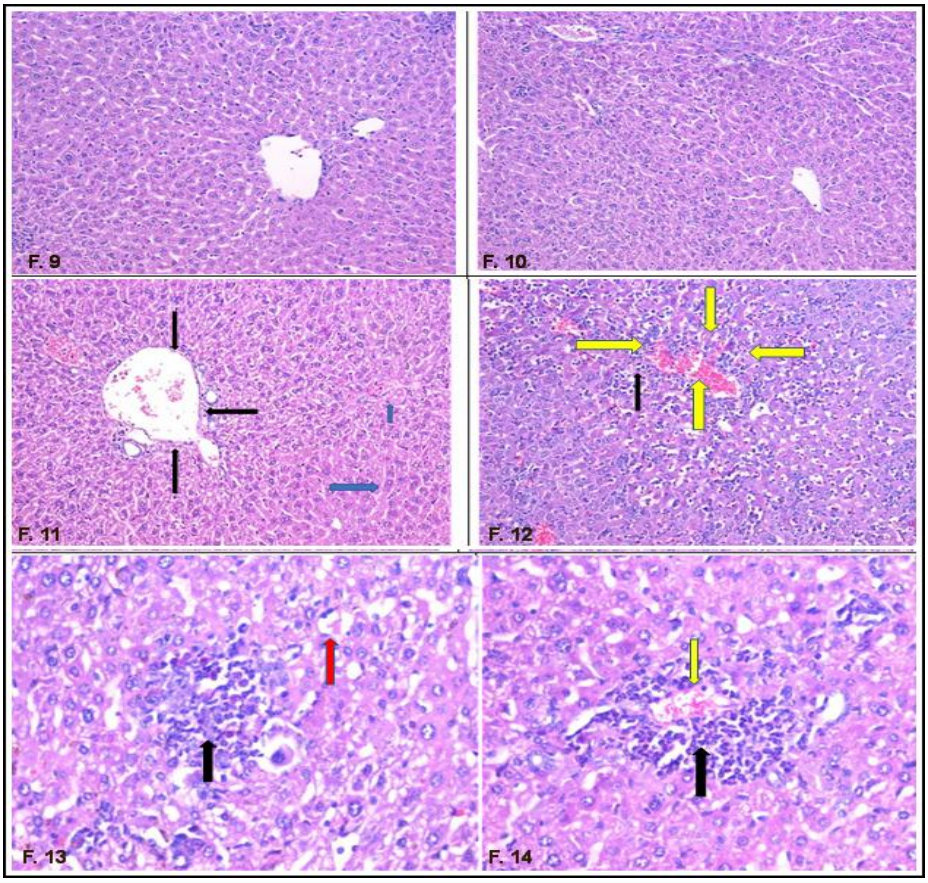
Fig. 5: Mouse brain treated with rosuvastatin (G IV) showed focal cerebral necrosis (blue arrow), neurofibrillary tangles (thin arrow), and shrinkage and degeneration cysts (black arrow) (H&E, x400).

Fig. 6: Mouse brain treated with spiramycin (GV) showed mild necrosis of neurons and neuronophagia (black arrow) (H&E, X400).

Fig. 7: Mouse brain treated with rosuvastatin (GVI) showed proliferation of glia cells (diffuse gliosis) (thin arrow) and minimal interstitial edema (black arrow) (H&E, X400).
Fig. 8: Mouse brain treated with spiramycin and rosuvastatin (GVII) showed minimal neurophagia and minimal necrosis of neurons (black arrow) and degeneration cysts (orange arrow) and apparently normal brain tissue (H&E, X400).
Fig. 9 &10: Mouse liver from control (GI) showed normal histological structure (H&E, X400).
Fig. 11: Mouse liver from positive control (GII) showed sever hydropic degeneration of hepatocytes (blue arrow) and lymphocytic infiltration (black arrow) and disturbed hepatic lobular architecture (H&E, X400).
Fig. 12: Mouse liver from infected non treated group (GII) showed severe lymphocytic infiltration (yellow arrow) and loss of hepatic lobular architecture (black arrow) (H&E, X400).
Fig. 13: Mouse liver treated with spiramycin (GIII) showed infiltration by large number of polymorph nuclear leucocytes (black arrow) and cloudy swelling with hydropic degeneration of hepatocytes (red arrow) (H&E, X400).
Fig. 14: Mouse liver treated with rosuvastatin (GIV) showed portal tract infiltration by large number of polymorph nuclear leucocytes (black arrow) and congestion of hepatic blood vessels (yellow arrow) (H&E, X400).
Fig. 15: Mouse liver treated with spiramycin (GV) showed congestion of hepatic blood vessels (black arrow), mild hydropic degeneration of hepatocytes (red arrow) and proliferation of polymorph nuclear leucocytes (yellow arrow) (H&E, X400).
Fig. 16: Mouse liver treated with rosuvastatin (GVI) showed infiltration of portal tract by large number of polymorph nuclear leucocytes (black arrow) (H&E, X400).
Fig. 17: Mouse liver treated with spiramycin and rosuvastatin (GVII) showed normal preserved hepatic lobular architecture, normal central vein without sinusoidal infiltration (black arrow) and minimal portal infiltration (blue arrow) (H&E, X400).



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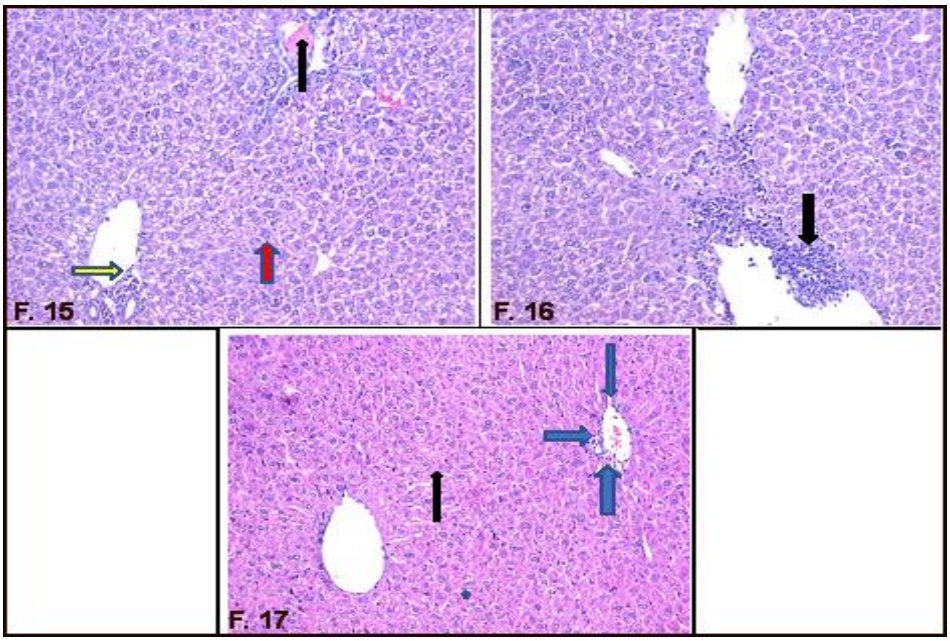
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