Original Article

The Histopathological Effect of Sofosbuvir as Compared to Sofosbuvir Combined with Ribavirin on Submandibular Salivary Glands of Adult Albino Rats (Histological and Immunofluorescent Study)

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ABSTRACT

Introduction: Sofosbuvir is one of the direct acting antiviral drugs (DAAs) acting on specific Hepatitis C virus (HCV) target proteins which are important for virus replication and assembly. Sofosbuvir can be used alone or combined with ribavirin which is a purine nucleoside analogue with antiviral activity against a wide range of RNA viruses. Ribavirin has a little antiviral activity against HCV when used alone.

Aim of Study: The present study aims at investigating the histopathological effect of sofosbuvir as compared to sofosbuvir used in combination with ribavirin on submandibular salivary glands of adult male albino rats.

Materials and Methods: 21 male albino rats were classified into 3 groups. Control group consisting of 5 rats, group A consisting of 8 rats which received sofosbuvir at a dose of 40 mg/kg/day for 5 weeks and group B consisting of 8 rats which received sofosbuvir at a dose of 40 mg/kg/day and ribavirin at a dose of 30mg/kg/day for 5 weeks. At the end of the experiment, submandibular glands were dissected and examined histologically and by immuno-fluorescent microscope.

Results: The submandibular salivary gland in rats treated with sofosbuvir (group A) showed degenerative effects, while those in group B showed more degenerative effects. Immunofluorescent expression of immunoglobulin M (IgM) was more pronounced in group B as compared to group A.

Conclusion: Treatment with sofosbuvir had a degenerative effect on submandibular gland which got worsen when used in combination with ribavirin. In addition, the immunoglobulin M expression was clear-cut in the group received both drugs.

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Key Words: HCV, IgM and submandibular salivary gland, ribavirin, sofosbuvir.

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INTRODUCTION

Hepatitis C virus (HCV) is an infectious virus that causes severe liver damage[1]. HCV is the main cause of death world-wide[2]. Egypt has the highest rate (15 percent -20 percent) of the virus. About 3–4 million individuals are affected by HCV yearly[3]. A series of (DAAs) was developed to overcome the adverse effects caused by interferon –based treatment. DAAs act on specific HCV target proteins which are important for virus replication and assembly. Therefore, treatment changed from IFN-based to IFN-free combinations, which is more effective and has better tolerability and protection[4].

Sofosbuvir, a nucleotide HCV polymerase inhibitor, due to its strong antiviral efficacy, pangenotypic activity, high barrier to resistance, favourable pharmacokinetics, and excellent tolerability and safety profile, it is considered the backbone of most DAAs combinations[5].

Ribavirin is a purine nucleoside analogue with antiviral activity against a wide range of RNA viruses. A great improvement in treatment of HCV was obtained when ribavirin was combined with Sofosbuvir[6].

This study aimed to assess the histological changes and immune-fluorescent expression of IgM of albino rat's submandibular salivary glands after administration of sofosbuvir alone or in combination with ribavirin.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee, Faculty of Dentistry, Cairo University, Cairo , Egypt. Committee approval number CU-III-F4518.

Materials

Animals

Twenty one adult male albino rats, with average weight of 300-400gm and with average age 18 months old were used in this study. The animals were housed into sterile, controlled environment (temperature 25 ± 2° and 12 hr dark/light cycles) and fed with standard pellets diet and tap water ad libitum. Each group was kept in separate stainless steel cages. All experiments were conducted in the animal house of the Faculty of Medicine, Cairo University according to the recommendations and approval of the ethics committee on animal's experimentation of the Faculty of Oral and Dental Medicine, Cairo University.

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Chemicals

Sofosbuvir: Each tablet contained 400 mg of Sofosbuvir, which was dissolved in distilled water and administered orally to rats through a gastric tube at a dosage of 40 mg/kg per day for 5 weeks[7].

Ribavirin: The usual dose is 200 mg tablet. Rats received an oral dose of 30 mg/kg per day via oral gavage[8].

Animals' design

Rats were divided into three groups as follows:

1. Control group: This group consisted of five rats which received distilled water via oral gavage for 5 weeks.
2. Group A (sofosbuvir- treated group): This group consisted of eight rats which received sofosbuvir in a dose 40 mg/kg/day dissolved in distilled water via oral gavage for 5 weeks
3. Group B (sofosbuvir- and ribavirin-treated group): This group consisted of eight rats which received sofosbuvir in a dose 40 mg/kg/day and ribavirin in a dose of 30 mg/kg/day dissolved in distilled water via oral gavage for 5 weeks.

Samples preparation

The animals were sacrificed 5 weeks after treatment with an intra-peritoneal injection of 100 mg/kg ketamine, and the submandibular glands were dissected out. The submandibular glands were washed in saline solution and fixed in 4 % buffered formalin for 24 hours before being dehydrated in ethyl alcohol in ascending grades, cleared in xylene, and embedded in paraffin. On positively charged microscope slides, 4-5 µm thick sections were obtained and collected. Until histological staining and immune-labeling, tissue parts were de-paraffinized and rehydrated.

Methods

LM examination using Hematoxylin and Eosin stain (H&E)

Specimens were immediately fixed in 10% neutral formalin for 48 h, washed, dehydrated in ascending grades of alcohol, embedded in paraffin and sectioned at 4-5 µm in thickness. They were conventionally stained with Hematoxylin and Eosin for histopathological examination[9]

Immunofluorescence Examination

For immune-fluorescent expression of IgM.

Procedures

Sections from each group were labeled using direct immunofluorescence technique. The sections were deparaffinized in xylene, rehydrated in descending grades of ethanol, and then held in phosphate buffered saline (PBS) for 2 hours at 56 °C in a hot oven. At 20-minute microwave, retreatment with a freshly prepared buffer containing EDTA pH 9.0 was performed. Then, the slides were cooled to room temperature before being incubated with rabbit antihuman antibodies (DAKO) labelled with polyclonal fluorescein isothiocynate (FITC): IgM (dilution 1:20). Incubation with the antibody was done in a moist chamber overnight at 4 °C. Incubation slides with PBS in the absence of the primary antibody were used as negative controls, which often produced negative results. All sections were placed in aqueous medium and analyzed using an ultraviolet fluorescence microscope with a dark field (Olympus-BX41)[10].

Histomorphometric Analysis

The histopathological changes in submandibular salivary glands were evaluated histomorphometrically in term of number of acini per field and number of nuclei per acini. An image analyzer device with the program Leica Quin 500 (Leica Microsystems, Switzerland) was used at The Oral Pathology Department, Faculty of Oral and Dental Medicine, Cairo University. A colored video camera, a colored display, and an IBM personal computer hard disc are attached to a microscope, making up the image analyzer. To determine the number of acini/field, we counted the acini in one field. For each group, we used 5 representative photographs of H&E-stained sections for analysis.

Statistical analysis

Data from histomorphometric analysis were represented as mean and standard deviation (SD) values. Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc Tukey test.

RESULTS

Histological Results

H&E

Control Group (CG)

Histological sections of this group showed normal structure of rat submandibular salivary gland, mainly serous secretory end portions. The serous acini have pyramidal shaped cells with basally situated nuclei (Figure 1b). The duct system consists of intercalated, striated, and excretory ducts, as well as granular convoluted tubules (GCTs). The intercalated duct was lined by a single layer of low cuboidal epithelial cells with centrally placed nuclei. The lining of the striated ducts was columnar cells with central nuclei and clear basal striations (Figure 1b). The epithelium of excretory duct consists of columnar cells. The GCT was located between the intercalated and striated ducts in the rat submandibular salivary gland. The GCT wall was composed of a simple columnar epithelium containing many secretory granules in its cytoplasm. (Figures 1a,b)

Group A

The submandibular gland of this experimental group showed atrophy of serous acini with very few acini with intra-cytoplasmic vacuolization (Figure 1c). The striated ducts and granular convoluted tubules exhibited obvious vacuolization and partial degeneration (Figures 1c,2b). Furthermore, mild atrophy of striated ducts and granular convoluted tubules was noticed. The granular convoluted tubules showed slight reduction in the content of eosinophilic granules (Figure 1c). The excretory ducts showed discontinuity of the epithelial lining and retained secretion with vacuolization of the duct (Figure 1c). Blood vessels were congested and dilated. The surrounding connective tissue septa was thickened with infiltration of chronic inflammatory cells (Figure 2a).

Group B

Sections of the submandibular gland of this group showed degeneration of serous acini, increased intra-cytoplasmic vacuolization of serous acinar cells, and shrinkage in the overall size of the serous acini (Figure 2d). Striated ducts and granular convoluted tubules were also vacuolized and were partially degenerated (Figure 2d). Granular convoluted tubules lost some of their eosinophilic granules. The excretory ducts showed thinning and loss of continuity of the epithelial lining, hyperplasia and vacuolization (Figure 2d). The blood vessels were dilated and congested with thickened walls (Figure 1d). The connective tissue stroma showed marked fibrosis with many chronic inflammatory cellular infiltration and marked widening of connective tissue septa was also noticed (Figures 1d,2c).

Immuno-fluorescent Results of Anti-IgM Anti-body Expression

Control Group (CG)

Immunofluorescence staining of the rat submandibular gland of control group showed weak to moderate immunoreactivity for IgM throughout the gland (Figure 4a). IgM-producing cells (plasma cells), scattered between acini, ducts and in the connective tissue stroma of the gland were detected, Plasma cells, with brightest immunofluorescence staining, were observed in many specimens (Figure 4a). Weak immunofluorescence staining was seen in the basement membrane of serous acinar cells, granular convoluted tubules and blood vessels (Figure 3a). Clear or negative immunofluorescence staining of the cytoplasm in the cells lining the acinar portion was observed. Also, a less intense immunofluorescence staining was seen in the intercalated, striated and excretory ducts of the gland (Figure 3a,4a).

Group A

Immunofluorescence staining of the rat submandibular gland of group A showed weak to strong immunoreactivity for IgM throughout the gland. Plasma cells were found in groups between acini, ducts and in the connective tissue stroma of the gland (Figure 3b). Plasma cells with brightest immunofluorescence staining was observed in many specimens (Figure 3b). Moderate immunofluorescence staining was seen in the periphery of serous acinar cells, granular convoluted tubules and the blood vessels. Also a moderate immuno-fluorescence staining was seen in the intercalated, striated and excretory ducts of the gland (Figure 4b). A stronger immunofluorescence staining with IgM in the apical cytoplasm of some excretory duct cells underneath the luminal border of the duct was detected (Figures 3b,4b)

Group B

Immunofluorescence staining of the rat submandibular gland of group B showed moderate to strong immunoreactivity for IgM throughout the gland. Moderate to strong immunofluorescence staining was seen in the basement membrane of serous acinar cells, granular convoluted tubules and the blood vessels (Figures 3c,4c) A stronger immunofluorescence staining of the periphery of the acinar portion was observed. Also a strong immuno-fluorescence staining was seen in the intercalated, striated and excretory ducts of the gland. A stronger immunofluorescence staining with IgM in the excretory duct cells was detected with plasma cells in the surrounding connective tissue (Figure 3c,4c).

Statistical Results

Statistical Analysis of Number of Serous Acini per Field

There was statistically significant difference between group B in comparison to control group and group A, where there was a significant decrease in the number of acini in group B than group A and control group. The *p-value* was < 0.001 (Figure 5a) (Table 1).

Statistical Analysis of Number of Nuclei per Acini

There was statistically significant difference between group B in comparison to control group and group A, where there was a significant decrease in the number of nuclei in group B than group A and control group. The *p-value* was < 0.001 (Figure 5b) (Table 2).

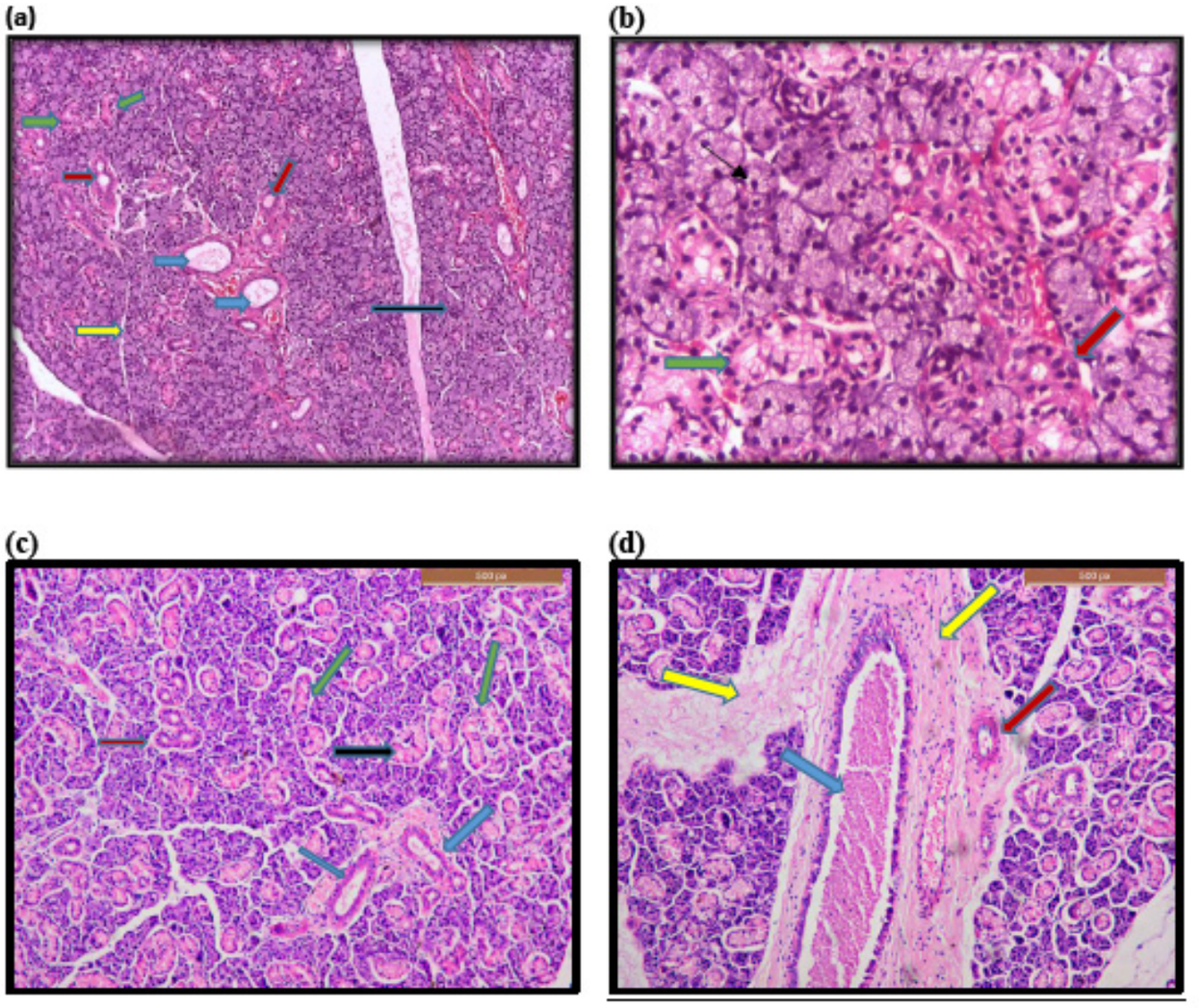

Fig. 1: Photomicrographs of submandibular salivary glands showing: (a &b) - CG: normal structure of the gland with serous acini (black arrow), granular convoluted tubules (green arrows), striated ducts (red arrows) , excretory ducts (blue arrows) and narraw C.T septa (yellow arrow). (c)-group A: atrophy of serous acini, striated ducts (red arrow) and granular convoluted tubules (green arrows) with slight reduction in contetnt of eosinophilic granules (black arrow), and some retained secretion of excretory duct (blue arrow). (d)-group B: retained secretion in excretory duct (blue arrow), increased width of C.T septa with increase in fibrous tissue amount (yellow arrow) and thickened walls of blood vessels (red arrow). (H&E, original magnification, a,c&d x100, bx400).

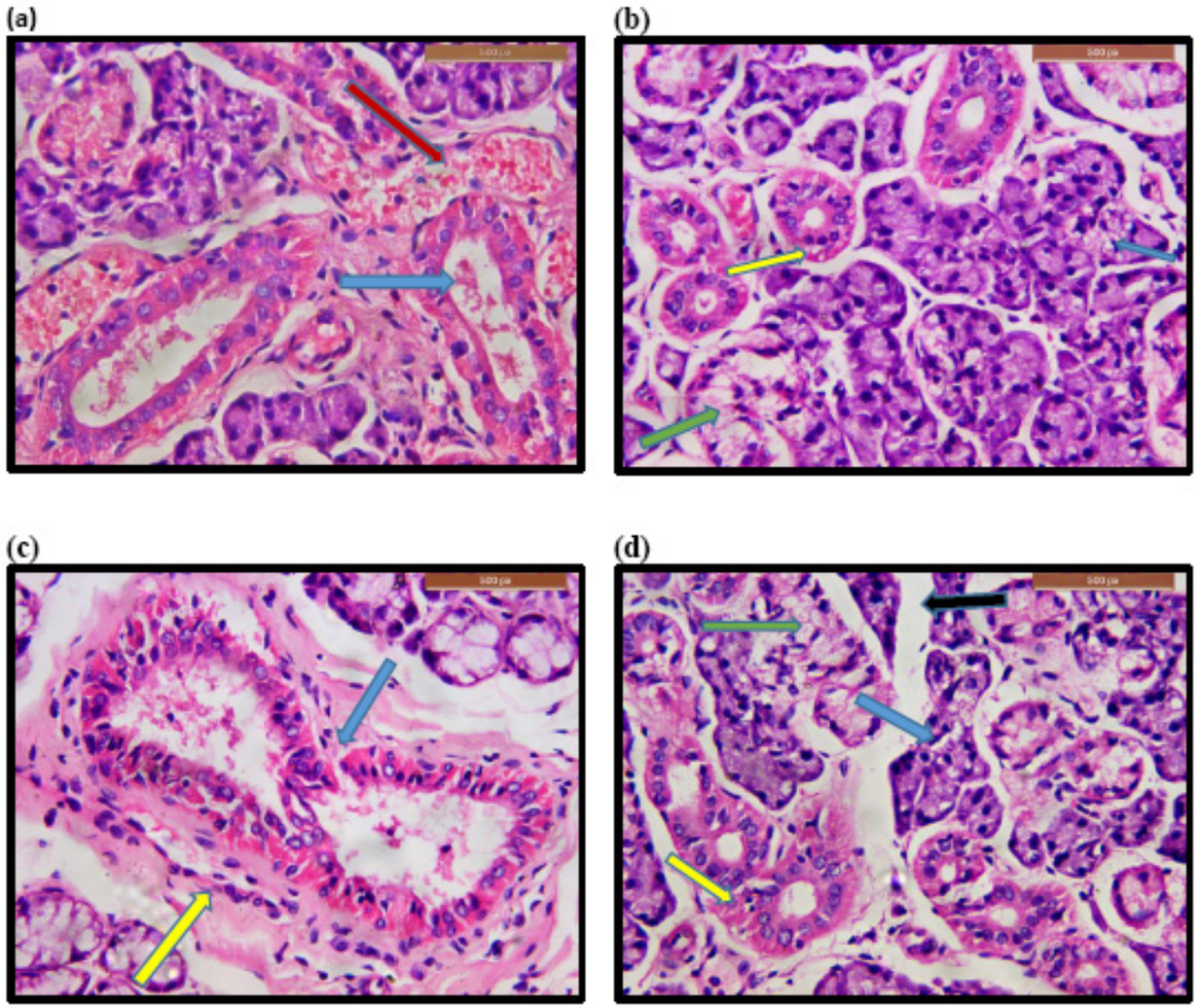

Fig. 2: Photomicrographs of submandibular salivary glands showing: (a)-group A: congeśtion of blood vessels (red arrow), traces of secretory material in excretory duct (blue arrow). (b)-group A: cytoplasmic vacuolization of serous acini (blue arrow), śtriated ducts (yellow arrow) and the granular convoluted tubules with reduced eosinophilic granules content (green arrow). (c)-group B: Degeneration and loss of continuity of the epithelial lining of excretory duct (blue arrow), and increased width of fibrous C.T septa with chronic inflammatory cells infilteration (yellow arrow). (d)-group B: Increased width of C.T septa (black arrow), vacuolization of serous acini (blue arrow), śtriated ducts (yellow arrow) and granular convoluted tubules (green arrow). (H&E, original magnification, a,b,c&dx400).

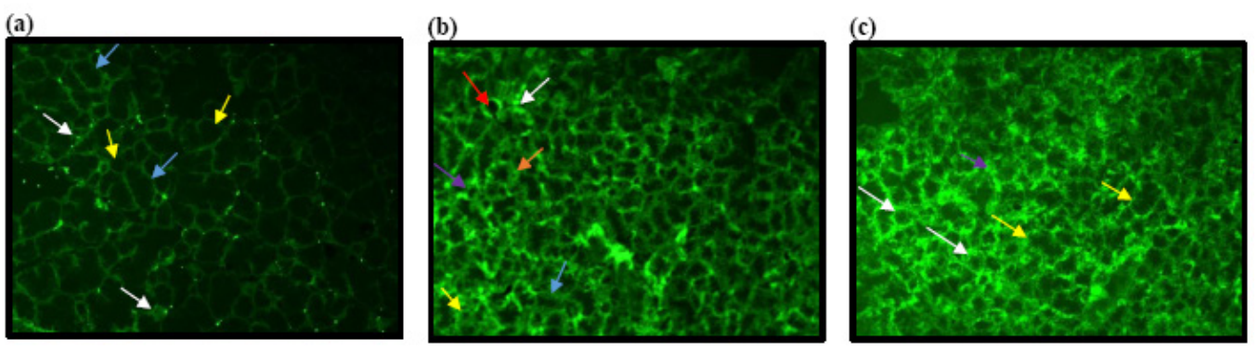

Fig. 3: Photomicrographs of submandibular salivary glands showing: (a)-control group: weak IgM immunoreaction in blood vessels (white arrows) and basement membrane of epithelial elements of duct syśtem and serous acini (yellow arrows) and granular convoluted tubules (blue arrows). (b)-group A: moderate IgM immunoreaction in śtriated duct (purple arrow), intercalated duct (orange arrow), serous acini periphery (yellow arrow) and GCTs (blue arrow), śtronger IgM immunoreaction in lumen of excretory duct (red arrow) plasma cells (white arrows). (c)-group B: śtrong IgM immunoreaction in śtriated duct (white arrows) serous acini periphery (purple arrow) and GCTs (yellow arrows). (FITC, original magnification, X100)

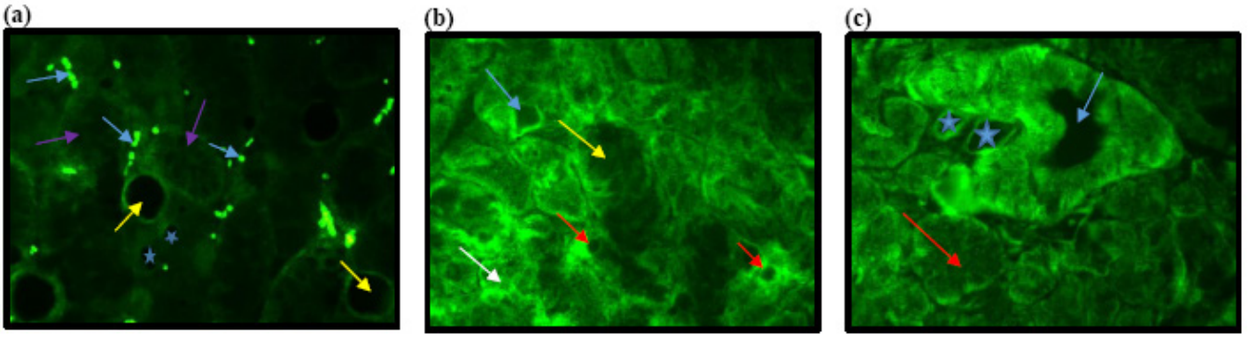

Fig. 4: Photomicrographs of submandibular salivary glands showing: (a)-control group: plasma cells scattered between acini and ducts (blue arrows) , negative to weak IgM immunoreaction in granular convoluted tubules (purple arrows) , negative IgM immunoreaction in blood vessels(blue stars) and moderate immunoreaction in lumen of excretory duct (yellow arrows). (b)-group A: moderate IgM immunoreaction in granular convoluted tubules (yellow arrow), striated duct (red arrows), serous acini (white arrow) and stronger IgM immunoreaction in excretory duct (blue arrow). (c)-group B: strong IgM immunoreaction in serous acini (red arrow), and blood vessels (blue stars) surrounding excretory duct (blue arrow). (FITC, original magnification, X400)

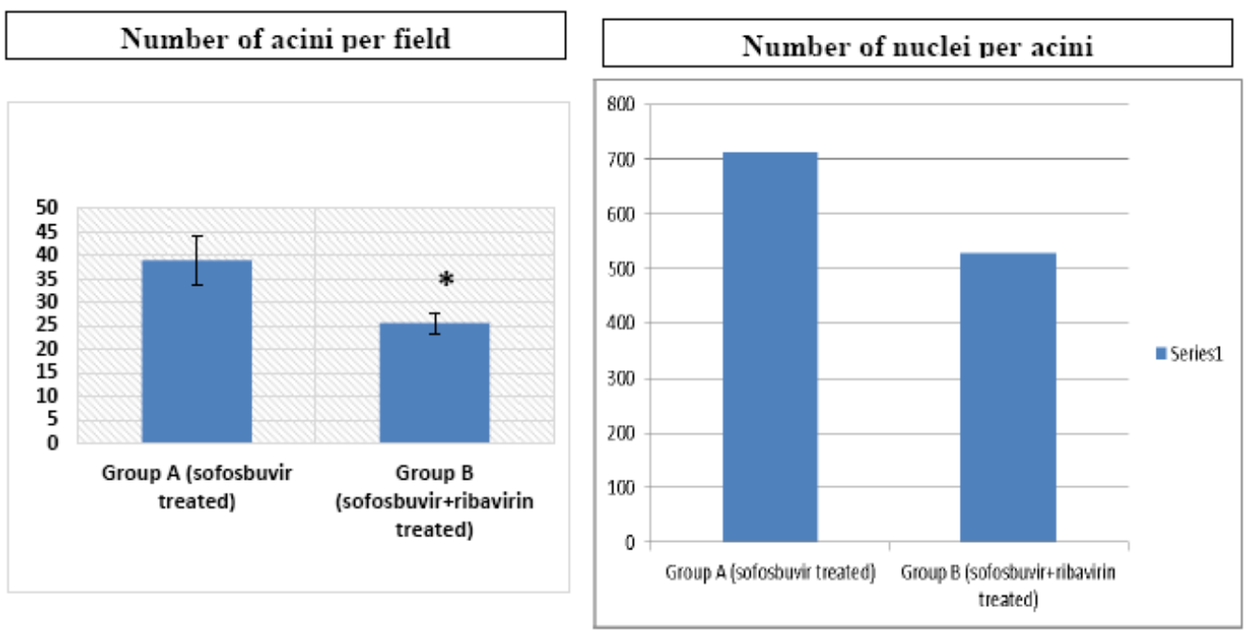

Fig. 5: Bar chart showing mean and SD values of (a)- number of acini per field in group A & group B (b)- number of nuclei per acini in group A & group B.

Table 1: Comparison between mean value of the number of acini per field by histomorphometric analysis among studied groups

	Group A	Group B	*P value*
Acini per field	38.9±5.16	25.5±2.2*	0.006

Table 2: Comparison between mean value of the number of nuclei per acini by histomorphometric analysis among studied groups

	Group A	Group B	*P value*
No of nuclei per acini	713.2 ±13.96	528.6± 7.76	0.005

DISCUSSION

Hepatitis C virus (HCV) infection is a significant cause of chronic liver disease around the world, with an estimated prevalence of 2.2 %, which represents approximately 170 million infected people worldwide[11].

Sofosbuvir is an oral drug which inhibits NS5B polymerase which is a non-structural protein important for replication of virus RNA[12,13,14]. Gane *et al.*[15] discovered that sofosbuvir was generally well tolerated either in interferon-based therapy and ribavirin or sofosbuvir monotherapy.

Albino rats were used in the current study as they have been recognized as the preeminent model mammalian system, showing histological similarity to human with shorter life span[16]. In addition, the choice of male over female was to exclude hormonal and other histological variations associated with the use of female rats[17]. The submandibular gland was used in the current study as it's responsible for the production of 65% of un-stimulated saliva[18].

The result of the present study demonstrated changes in the histological pattern of the salivary gland in the experimental groups. Group A showed little vacuolization of acini. Furthermore, the striated ducts and GCTs exhibited obvious vacuolization and were partially degenerated. Discontinuity of the epithelial lining of the excretory ducts was noticed with retained secretion. Blood vessels were congested with chronic inflammatory cellular infiltration in the surrounding connective tissue. These findings were similar to the findings of Salem *et al*.[19] who found that increased levels of mitochondrial reactive oxygen species (mROS) caused obvious immune expression of NFκB, cellular vacuolization and distortion in the basement membrane of some acini and granular ducts of their experimental groups as a result of sofosbuvir administration.

These changes were accentuated in group B, where shrinkage in the overall size of the serous acini, striated ducts and GCTs showed vacuolization, complete degeneration in some of the acini and ducts. Thinning and loss of continuity of the epithelial lining of excretory ducts and vacuolization were observed .The blood vessels were dilated and congested with increased chronic inflammatory cells. These findings were thought to occur due to the combination of sofosbuvir and ribavirin which leaded to the worse histological picture as a result of cell damage. Aghemo *et al*. showed that Ribavirin caused hypoplasia and xerostomia resulting from a reversible inhibition of salivary gland function[20,21] which supported the current study.

In the current study, the eosinophilic granular content of GCTs was reduced. This could be explained by Gresik[22] who found that this was consistent with functional decline indicated by decrease in the epidermal growth factor.

Abdeen *et al*.[23] study coincided with the present study where serous acini showed swelling and vacuoliztion after sofosbuvir administration. Mild vacuolization of granular convoluted tubules and striated ducts was observed as well as less compactness of the gland which denoted increased oedema.

Regarding retained secretion of excretory ducts as well as areas of flattening in their epithelial lining. This could be explained according to Parlak *et al*.[24] who suggested that this flattening may occur due to metaplasia of the ductal cells along with the accumulated secretion and secondary to glandular injury.

Histological results of the present study also revealed widening of connective tissue septa which was similar to the finding reported by Issa and El-Sherif[25] who discovered after 5 weeks of sofosbuvir treatment, focal areas of desquamation, separation, and large spaces in the corneal epithelium, suggesting corneal oedema.

The present study revealed degeneration of acini which caused decreased salivary flow. This was in agreement with the results of Mahboobi & Haghighi[26] who concluded that ribavirin monotherapy had a direct role in transient inhibition of salivary gland function causing xerostomia.

In terms of blood vessel dilation and congestion, and mononuclear cellular infiltration, these findings were consistent with those of Issa and ElSherif[25], who found vascular dilatation and RBCs extravasation from cerebral cortex blood vessels after 5 weeks of SOF administration.

Some salivary proteins, such as immunoglobulins A, G and M were secreted by acinar cells and had a significant role in oral cavity protection[27]. The present study showed an increase in immuno-fluorescent expression of immunoglobulin M in group B as compared by group A which suggested that IgM levels increased with salivary gland degeneration.

Brandtzaeg explained the presence of high levels of the IgM in whole saliva as a result of crevicular leakage as its level was significantly related to both the serum IgM concentration and periodontal inflammation[28]. In contrast, Amor & Kahan[29] concluded that synthesis of IgM was related to the degree of lymphoid infiltration but not with serum immunoglobulin concentration. In the current study group B showed increased chronic inflammatory cells together with strong immuno-expression of IgM which agreed with Amor & Kahan.

The current study showed an increased level of IgM in group B as compared to group A which agreed with Bergmann[30] and Matos-Gomes *et al*.[27] who found that the concentration of immunoglobulins increased when salivary flow rate decreased and vice versa.

Sozmen *et al*.[31] found that IgM-containing plasma cells were increased with periductal and interlobular plasma cell infiltration due to dog's submandibular gland inflammation. In the current study, IgM-containing plasma cells were increased with chronic inflammatory cells infilteration and salivary gland damage which agreed with Sozmen *et al*.[31].

CONCLUSION

The overall results of the current study, clearly ascertain that sofosbuvir caused degenerative effects on submandibular salivary gland structure which was worsen by the combination therapy of sofosbuvir with ribavirin. These alterations of the submandibular salivary gland were considered as a sign of toxicity which leaded to the worse histological picture as a result of cell damage.

Therefore, the present study concluded that sofosbuvir combined with ribavirin caused more degenerative effects on the submandibular gland structure causing increased level of IgM.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثيرالهيستوباثولوجي لعقار السوفوسبفير مقارنة بالسوفوسبفيرمع الريبافيرين على انسجة غدد تحت الفك للفئران البيضاء البالغة (دراسة هستولوجية ومناعية بواسطة ميكروسكوب فلوري)

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الخلفية: فيروس التهاب الكبد الوبائي (سي) هو فيروس معدي يسبب تلفًا حادًا في الكبد وموتًا لاحقًا. تم تطوير سلسلة من العوامل المباشرة المضادة للفيروسات لعلاج التهاب الكبد الوبائي. سوفوسبوفير هو أحد العوامل المباشرة المضادة للفيروسات الذي يعمل على بروتينات مستهدفة محددة لفيروس سي والتي تعتبر مهمة لتكاثر الفيروس وتجميعه. يمكن استخدام سوفوسبوفير بمفرده أو مع ريبافيرين وهو نظير نيوكليوزيد البيورين مع نشاط مضاد للفيروسات ضد مجموعة واسعة من فيروسات الحمض النووي الريبي.

هدف الدراسة: لمعرفة التأثير الهستولوجي المرضي لسوفوسبوفير مقارنة بالسوفوسبوفير مع ريبافيرين على الغدد اللعابية تحت الفك السفلي للجرذان البيضاء البالغة.

طرق البحث: تم تصنيف ٢١ من ذكور الجرذان البيضاء إلى ٣ مجموعات. المجموعة الضابطة المكونة من ٥ جرذان، المجموعة أ المكونة من ٨ جرذان تلقت سوفوسبوفير بجرعة ٤٠ ملجم / كجم / يوم لمدة ٥ أسابيع والمجموعة ب المكونة من ٨ جرذان تلقت سوفوسبوفير بجرعة ٤٠ مجم / كجم / يوم وريبافيرين بجرعة الجرعة ٣٠ مجم / كجم / يوم لمدة ٥ أسابيع. في نهاية التجربة ، تم تشريح الغدد تحت الفك وفحصها هستولوجيا بواسطة الميكروسكوب الضوئي ومناعيا بواسطة الميكروسكوب الفلورسنتي.

النتائج: في المجموعة الضابطة ، أظهرت النتائج الميكروسكوبية الضوئية التركيب الطبيعي للغدة اللعابية تحت الفك السفلي. أظهرت المجموعة (أ) تأثيرات تنكسية وأظهرت المجموعة (ب) تأثيرات تنكسية أكثر على الغدد اللعابية تحت الفك السفلي. أوضحت النتائج زيادة التعبير المناعي للاجسام المضادة مـ في المجموعة ب مقارنة بالمجموعة أ.

الاستنتاج: كان للعلاج باستخدام سوفوسبوفير تأثير تنكسي على الغدة تحت الفك السفلي والذي ساء عندما تم دمج سوفوسبوفير مع ريبافيرين مما أظهر زيادة في تعبير للاجسام المضادة مـ.