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EFFECT OF AN ANTI-HEPATITIS C VIRAL DRUG ON RAT SUBMANDIBULAR SALIVARY GLAND

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ABSTRACT

Background: In the last 3 years, hepatitis C virus (HCV) treatment has undergone significant changes. Sofosbuvir is a new drug; it is a nucleotide analogue that is a highly potent inhibitor of the nonstructural protein 5B (NS5B) polymerase in HCV.

Methodology: 30 adult male albino rats weighing about ±200 gm were used. The rats were divided into three groups 10 rats each. ***Group I(control group)*** received distilled water. ***Group II*** received sofosbuvir 40 mg/kg/day dissolved in distilled water for 45 days. **G*roup III*** received sofosbuvir 40 mg/kg/day dissolved in distilled water for 90 days. All animals were sacrificed by ketamine overdose. The submandibular salivary glands were dissected. Specimens were examined histologically, immunohistochemically using anti- nuclear factor κ B antibody, histomorphomotry and statistically.

Results: Group I showed normal histological structure for the ducts and acini however, group II showed acinar and ductal vacuolization, discontinuity of the epithelial lining with retained secretion in the excretory duct, and chronic inflammatory cell infiltration. These changes were accentuated in group III accompanied by acinar and ductal shrinkage. **Immunohistochemical results** of group I showed negative immunoreactivity for NF-κB in nuclei of the acini and ducts with very weak cytoplasmic reaction. However group II showed moderate nuclear and cytoplasmic immunoreactivity for NF-κB in the acinar and ductal cells. Regarding group III, the ductal and acinar cells exhibited strong immunoreaction in their nuclei and cytoplasm. **Statistical results** proved that the difference in the mean values were statistically highly significant between groups II & I, groups III & I and groups II & III.

Conclusion: We concluded that sofosbuvir may affect the function of submandibular salivary gland through increased ROS levels in acinar and ductal cells leading to degenerative effect.

KEY WORDS: Sofosbuvir, submandibular salivary gland, histological study, NF-κB.

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INTRODUCTION

Hepatitis C, caused by different genotypes of the Hepatitis C virus (HCV), recently infects more than 170 million people world wise. it may lead to chronic hepatitis, decompensated cirrhosis, and hepatocellular carcinoma, causing as many as 350,000 deaths per year.[1]

In the last 3 years, hepatitis C virus (HCV) treatment has undergone severe changes. Direct acting antivirals(DAAs), when combined with pegylated interferon (PegIFN) and ribavirin (RBV), improved rates of healing in chronic HCV infection as compared to the use of PegIFN and RBV alone.[2]

Sofosbuvir (SOF) is a new drug candidate for hepatitis C treatment, with the chemical name L-Alanine, N-[[P(S),2′R]-2′-deoxy-2′-fluoro-2′-methyl-P-phenyl-5′-uridylyl]-, 1-methylethyl ester and a molecular formula of C22H29FN3O9P.[3] Previously known as PS-7977 [4] or GS-7977, it has shown promising results in numerous in-vitro studies against all the genotypes of HCV. It is a nucleotide analogue that is a highly potent inhibitor of the NS5B polymerase in HCV. This drug has shown high efficacy in combination with several other drugs with and without Peg-INF, against HCV.[5,6]

NS5B is one of the non-structural proteins essential for viral RNA replication, and has been found to be a valuable target for directly acting antiviral agents (DAAs).[7]

The uridine nucleotide analogue sofosbuvir is a phosphoramidate prodrug that has to be triphosphorylated within the cells to produce its action. The enzymes needed for its activation are located in the human hepatic cells, so, it is changed to its active metabolite during the first-phase metabolism, directly at the preferred site of action: the liver.[8] The active nucleoside triphosphate form GS- 461203 has a long half-life of approximately 18 h. It is irreversibly metabolized to the inactive phosphate free metabolite of the nucleotide, GS-331007. This metabolite is largely eliminated by passive filtration in the renal glomerulus.[9]

The active metabolite mimics the physiological nucleotide and competitively blocks the NS5B polymerase, thus inhibiting the HCV-RNA synthesis by RNA chain termination.[10,11]

Sofosbuvir (brand name Sovaldi TM) is administered once-daily as a 400 mg oral tablet without a food or fasting requirement. Sofosbuvir is absorbed with a peak plasma concentration observed at, 0.5–2 h. post-dose.[12,13]

From the above review, it seems that SOF is a promising therapy for chronic HCV infection; however its adverse effects in general and on oral tissues in specific had not been well documented. Thus, the aim of the present study is to investigate the possible effect of sofosbuvir on submandibular salivary gland in albino rats both histologically and immunohistochemically by using anti- nuclear factor κ B antibody.

MATERIAL & METHODS

Animals:

The study was conducted on 30 adult male albino rats weighing about ± 200 gm and obtained from the Animal house, Faculty of medicine, Cairo University, Egypt. The animals were kept in a 12-hour light/dark cycle, at a temperature of 22°C ± 2°C with relative humidity 50% ± 20%. They were fed on standard chow pellets and tap water *ad libitum* for the entire test period.

Experimental design:

The rats were randomly assigned into three groups, of 10 rats each.

Group I (control group): Served as control and the rats received distilled water through oral gavage.

Group II (experimental group): Received sofosbuvir* dissolved in distilled water (8mg/ml) through oral gavage in a dose of 40 mg/kg/day[14] for 45 days.

G*roup (III) (experimental group):* Received sofosbuvir* dissolved in distilled water (8mg/ml) through oral gavage in a dose of 40 mg/kg/day[14] for 90 days.

All animals were sacrificed by ketamine overdose and the submandibular salivary glands were dissected.

Light microscopic examination:

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 Heamatoxylin and Eosin for histopathological examination.

Immunohistochemical (IHC) examination:

Sections of 4μ thickness were cut and placed on positively charged slides, air dried for 30 minutes, then fixed in a 65˚C oven for 1 h. Slides were placed in a coplin jar filled with 60 ml of triology (Cell Marque, CA-USA. cat# 920p-06). The working solution and the jar were securely positioned in the autoclave. The autoclave was adjusted to 120 ˚C and maintained stable for 15 minutes after which pressure was released. Slides were then left to cool for 30 minutes. Sections were washed and immersed in TBS (Tris buffered Saline, Ameresco-USA) to adjust the PH, this was repeated between each step of the IHC procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 minutes.

Broad spectrum LAB-SA detection system from Invitrogen (Cat# 85-9043) was used to visualize any antigen-antibody reaction that may occur in the tissues. The background staining was blocked by 10% goat non immune serum blocker. Primary antibody (anti- nuclear factor κ B; NF-κB antibody) was added, and then the slides were incubated in the humidity chamber for 1 hour. Biotinylated secondary antibody was then applied on each slide for 20 minutes, followed by 20 minutes incubation with the enzyme conjugate. DAB chromogen was applied on each slide then the slides were counter stained, dehydrated and cleared to be finally mounted. The positive results were indicated by brown coloration of cell cytoplasm for NFκB.

Image Analysis:

For staining affinity evaluation, the immunoreactivity was measured by an image analysis system (Leica DM LB2 with QWIN Plus image analyzer computer system, Germany). The area percentage of NFκB was measured in the submandibular salivary glands in ten small measuring frames; in each specimen using an objective lens of magnification 400x. The image was transformed into a grey image, and then delineated to choose the areas exhibiting positive reactivity.

Statistical analysis:

Data were statistically described in terms of mean ± standard deviation (± SD), and range and compared using one way analysis of variance (ANOVA) test with posthoc multiple 2-group comparisons. *p* values less than 0.05 was considered statistically significant. All statistical calculations were done using computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) release 15 for Microsoft Windows (2006).

*Gratisovir® tablets, Pharco Pharmaceuticals, Amriya- Alexandria. (Sofosbuvir400mg)

RESULTS

Histological results:

Group I (control)

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

Group II:

Specimens of this experimental group showed very few acini with vacuolization. Furthermore, the striated ducts and granular convoluted tubules exhibited obvious vacuolization and partial degeneration (Fig. 2).The excretory ducts showed discontinuity of the epithelial lining and retained secretion (Fig. 3 A). Also, extensive fat cells were noticed in the connective tissue stroma (Fig. 3B).

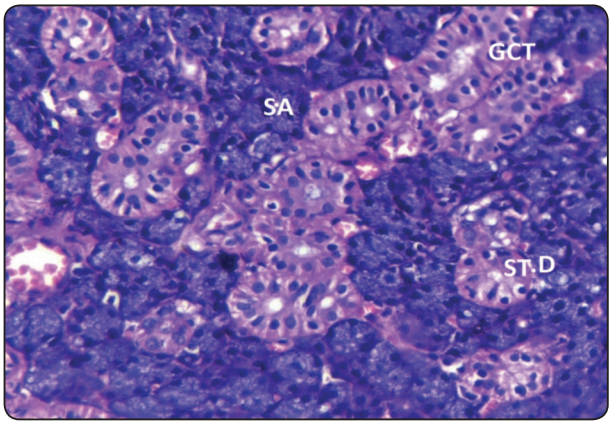

Fig. (1) A photomicrograph of submandibular salivary gland of rats of group I (control group) showing nearly normal structure of the serous acini (SA), striated ducts (ST.D) and granular convoluted tubules (GCT) .(H&E X400)

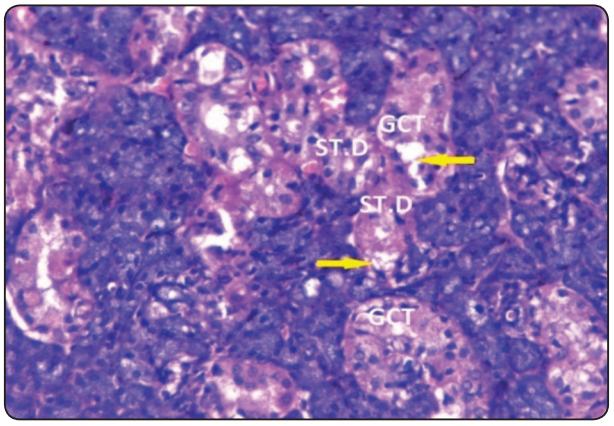

Fig. (2) A photomicrograph of submandibular salivary gland of rats of group II. It showed obvious vacuolization (arrows) in the GCT and striated ducts (ST.D). H& E orig. mag. X400

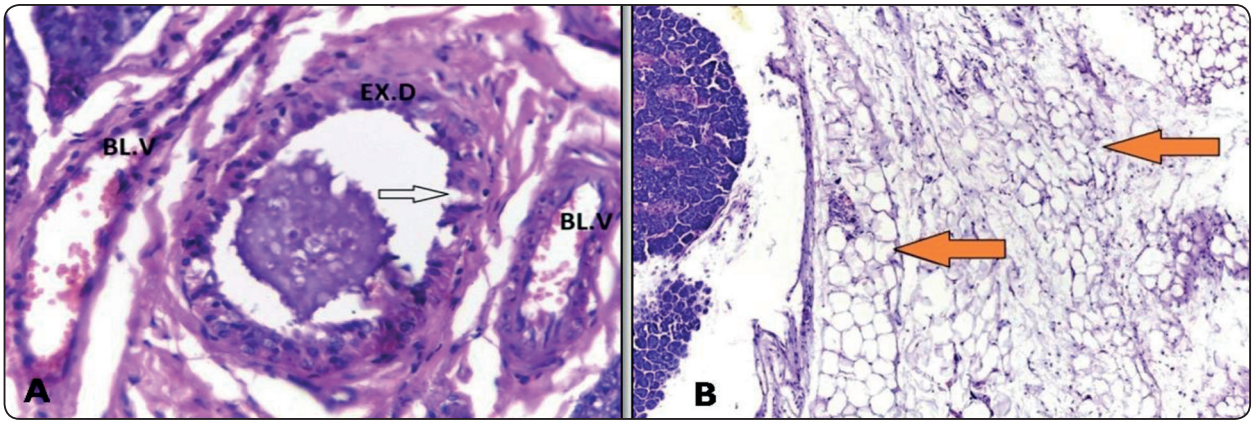

Fig. (3) A photomicrograph of submandibular salivary gland of rats of group II showing: (A) discontinuity of epithelial lining (White arrow) of the excretory ducts (EX.D) with retained secretion.Dilated blood vessels (BL.V). (B) Extensive fat cells areas (orange arrows) in the connective tissue stroma. H&E orig. mag. (A)X400, (B)X200.

Group III:

Specimens of this group showed vacuolization and shrinkage in the overall size of the serous acini, striated ducts and granular convoluted tubules (Fig. 4A). Also, some areas the acini and ducts exhibited partial degeneration. The excretory ducts showed thinning of the epithelial lining and vacuolization. The blood vessels were dilated and congested. Many inflammatory cells were observed in the connective tissue stroma (Fig. 4B).

Immunohistochemical results

In the control group (group I) negative immunoexpression of NFκB was observed in nuclei of serous acini, GCT and striated ducts of submandibular salivary gland, while the cytoplasm showed weak expression (Fig. 5 A). In experimental group II moderate NFκB immunoexpression was noticed in some nuclei of acinar, ductal and GCT cells of submandibular salivary gland, also the cytoplasm showed moderate expression (Fig.5B). Strong NFκB immunoreactivity in the cytoplasm and most of nuclei of acinar and ductal cells of the experimental group III was observed (Fig. 5C).

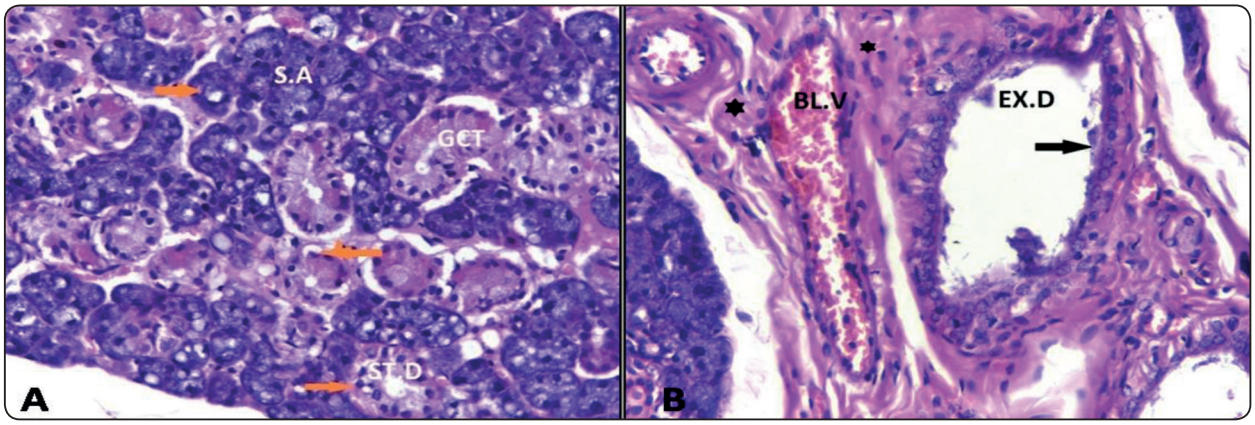

Fig. (4) A photomicrograph of submandibular salivary gland of rats of group III showing: (A) Noticeable vacuolization (orange arrows) and shrinkage in the overall size of serous acini (S.A), striated ducts (ST.D) and GCT. (B) Thinning of the epithelial lining (black arrow) and vacuolization of the excretory ducts (EX.D). Dilated and congested blood vessels (BL.V) with many inflammatory cells (black stars). H&E orig. mag. (A) X400, (B) X400

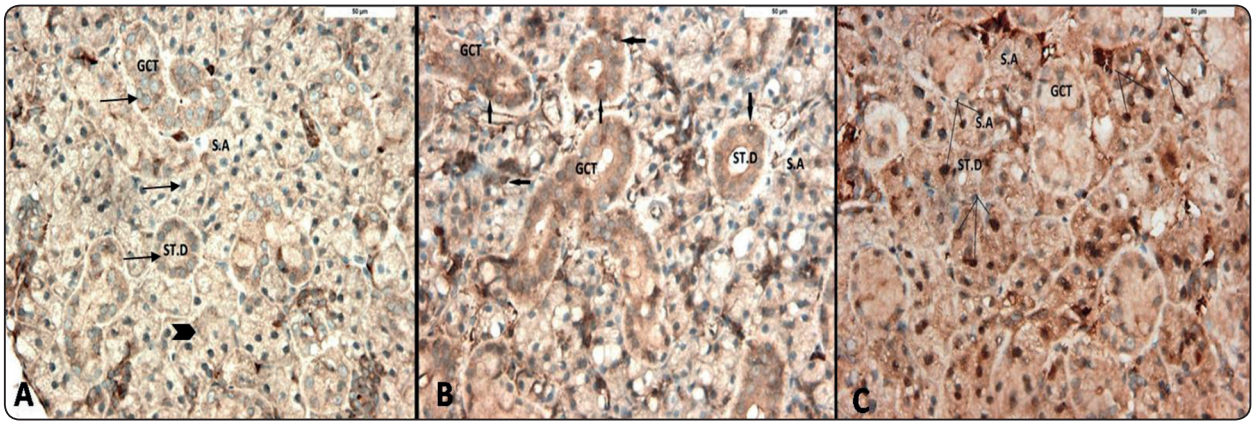

Fig. (5) A photomicrograph of submandibular salivary gland of rats of group (A) showing NFκB negative expression in nuclei (arrows) of acini, striated ducts and GCT cells. The cytoplasm (arrow head) showed weak NFκB immunoexpression. Group II (B) showing moderate NFκB expression (black arrows) in some nuclei of acini, striated ducts and GCT cells. The cytoplasm showed moderate NFκB immunoexpression. Group III (C) showing strong NFκB immunoexpression (black lines) in most of nuclei of acini, striated ducts and GCT cells. The cytoplasm showed strong NFκB immunoexpression. NFκB orig. mag.X400

Statistical analysis results

Data obtained from image analysis for NF-κB immune-expression among the three studied groups were expressed as means ± standard deviation **(Table 1).** The greatest mean value of area percentage of NF-κB immune-expression was recorded in group III, whereas the least value was recorded in group I. ANOVA test revealed that the difference was statistically highly significant (p-value ≤ 0.0001). Post hoc multiple 2-group comparisons revealed that, the difference in the mean values were statistically highly significant between groups II & I, groups III & I and groups II & III.(p-value≤ 0.05) **(Table 1).**

TABLE (1) Comparison of mean values (mean ± SD) of the area percentage for NF-κB expression for the three studied groups.

Studied groups	NF-κ B immunoexpression
Group I	0.5660 ±0.06914
Group II	1.8540 ±0.35536[1]
Group III	3.7420 ±0.96844[2]
P value*	(≤ 0.0001)

**** ANOVA test.***

1-Significant compared with group I (p ≤ 0.05);

2-Significant compared with groups I (P≤ 0.05),and II (P≤ 0.05)

(P-values from pair-wise comparisons of study groups using Post hoc test.)

DISCUSSION

Hepatitis C virus (HCV) is a global health problem, with an estimated 180 million individuals chronically infected with the virus[15]. The infection is the leading cause of end-stage liver disease and liver cancer in North America and Europe [16]. For the past two decades, interferon-dependent therapy has been the keystone of HCV treatment, but achievement has been limited by reduced tolerability and suboptimal sustained virological response (SVR) rates, even when combined with ribavirin[5].

SOF is an oral nucleotide inhibitor of the HCV nonstructural protein 5B (NS5B) RNA dependent RNA polymerase enzyme, and the agent demonstrates this activity across all genotypes of HCV[17].

In the current study, sofosbuvir dose was calculated so that it was equivalent to the clinically administered dose. The usual total adult dose of sofosbuvir per day is 400 mg [13]. According to Shin et al. [14], this dose was equivalent to 8 mg per day for a rat weighting about 200 gm.

Sofosbuvir as one of antiviral treatment may cause unwanted side effects that require medical attention. Very common (10% or more): Nausea (up to 34%), diarrhea (up to 12%), vomiting, ulcers, sores, or white spots in the mouth. Common (1% to 10%): Increased lipase, abdominal discomfort, constipation, dyspepsia, dry mouth and gastro-esophageal reflux [18,19].

The current study investigated the changes in the structure of submandibular salivary glands of rat histologically and immunohistochemically after SOF administration.

The result of the present study demonstrated changes in the histological pattern of the salivary gland in the experimental groups (Sofosbuvir treated groups). Group II showed acinar and ductal vacuolization, discontinuity of the epithelial lining with retained secretion in the excretory duct, and chronic inflammatory cell infiltration. These changes were accentuated in group III accompanied by acinar and ductal shrinkage.

Regarding the results of NF-κB, the immunostained sections of the rat submandibular salivary gland of group I showed negative

immunoreactivity for NF-κB in nuclei of the acini and ducts with very weak cytoplasmic reaction. These results are consistent with Zhang et.al. [20]. They found a slight staining of NF-κB in the cytoplasm of tubular cells in normal salivary glands, whereas there was no staining in the acinar cells.

However the present study showed in group II moderate nuclear and cytoplasmic immunoreactivity for NF-κB in the acinar and ductal cells. Regarding group III, the ductal and acinar cells exhibited strong immunoreaction for NF-κB in their nuclei and cytoplasm.

As the mitochondria play an important role in the generation of reactive oxygen species (mROS), which are produced physiologically during oxidative phosphorylation. Oxidative stresses occur if there is an imbalance between the production of ROS (an important indicator of oxidative stress) and cellular antioxidant defenses. Also, damaged mitochondria secrete higher levels of ROS [21]. Mitochondrial polymerase is a target of oxidative damage [22, 23].

Recent publications indicate that mitochondrial ROS serve as signaling molecules to activate pro-inflammatory cytokine production. The effects of inflammatory cytokines depend on complex and many-sided regulation to limit collateral damage to normal tissue. Although these are well-identified methods of regulating proinflammatory cytokine production, it is becoming obvious that an additional factor of complexity subsist as signals provided by ROS.[24-28]. More than a decade ago mROS were known to be involved in NFκB signaling activation. [29]

NF-κB fits in the category of "rapid-acting" primary transcription factors as it presents in cytoplasm in a latent form that can be rapidly activated without new protein synthesis. The activity of NF-κB is synchronized by an inhibitor of nuclear transcription factor NF-κB, known as IκBα. Activation of the typical NF-κB signaling pathway by TNF leads to in rapid phosphorylation and dissociation of IκBα from the complex with NFκB, which culminates in IκBα descentigration by a cytosolic proteasome[30] .

On the other hand, among the genes positively regulated by NFκB, there is inducible nitric oxide synthase (iNOS). iNOS can produce a huge amount of reactive oxygen species (ROS) in form of nitric oxide (NO) that can combine with superoxide anions to release the cytotoxic compound peroxy-nitrite responsible for lipid peroxidation and interstitial fibrosis[31 &32].

Nucleos(t)ide analogues that have great affinity for mitochondrial polymerases can lead to mitochondrial damage, and so, cell death. This may result in many of the toxicities that have been seen with members of this antiviral class [33].

So, in the present study the obvious immunoexpression of NFκB (gp II& III), cellular vacuolization and distortion that were observed in the basement membrane of some acini and granular ducts of group II, III could be attributed to the increased levels of mROS as a result of sofosbuvir administration. In accordance with our results **Schon & Przedborski 2011** [34] reported that over production of mROS induces cellular damage. Additionally, **Lalia et al., 2016** [35] demonstrated signs of degeneration in the acini and ducts of submandibular salivary glands of rat as a result of Cadmium induced oxidative stress

CONCLUSION

In this study, we demonstrated the effect of sofosbuvir on the structure of submandibular salivary glands. We concluded that sofosbuvir may affect the function of submandibular salivary gland through increased ROS levels in acinar and ductal cells leading to degenerative effect. Further researches are recommended with a more experimental and clinical trials on sofosbuvir effect on different dental tissues.

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