

Light and electronic histological studies to the effect of Sofosbuvir on the visual cerebral cortex of adult male albino rat

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Abstract: Sofosbuvir is a nucleotide analog that has activity against all hepatitis c virus genotypes. The aim of this study was to evaluate the effect of Sofosbuvir on the visual cerebral cortex in adult male albino rat. Twenty adult male albino rats were used in this experiment; each of them was weighing 150-200 grams. Animals were equally divided into two groups: Group I (Control) and Group II (Sovaldi treated) that received Sofosbuvir available in the form of tablets. Each tablet contained 400 mg of Sofosbuvir; these tablets were dissolved in distilled water and given for rats orally by gastric tube in a dose of 4mg/kg per day for 5 weeks. Examination of H&E stained sections of the visual cortex of Sovaldi treated rats showed hypercellularity. Neurons were distorted in shape and shrunken with deeply stained nuclei and surrounded by vacuolated pale areas. Ghost masses with deeply stained fragmented nuclei and surrounded by clear halos were present. Neuropil appeared vacuolated and showed vascular dilatation and extravasation of blood. Sovaldi treatment dramatically up-regulated the expression of Ki67 and glial fibrillary acidic protein (GFAP). Pyramidal and granular neurons exhibited irregular heterochromatic shrunken nuclei and peripheral clumping of chromatin. Cytoplasm appeared rarefied and showed degeneration of cellular organelles with cristolysis of the mitochondria. The axons exhibited irregular contour and multiple splitting of their myelin lamellae with wide spaces in between. Sofosbuvir treatment resulted in histological changes on the visual cerebral cortex.

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Keywords: Sofosbuvir, visual cerebral cortex, Ki67, GFAP.

1. Introduction:

Sofosbuvir is commercially known as Sovaldi, it is a nucleotide analog used in combination with other drugs to treat hepatitis C virus (HCV) infection. It has been marketed since 2013. On comparing Sofosbuvir-based regimens to previous treatments, it provides a higher cure rate, fewer side effects, and a two- to four-folds reduced duration of therapy[1]. Sofosbuvir has allowed most patients to be treated successfully without need to use peginterferon, an injectable drug with severe side effects that is a key component of older drug regimen for the treatment of HCV [2]. Sofosbuvir is a prodrug using the ProTide biotechnology strategy. It is converted to the active antiviral agent 2'-deoxy-2'- α -fluoro- β -C-methyluridine-5'-triphosphate. The triphosphate acts as a defective substrate for the NS5B protein, which is the viral RNA polymerase, thus acts as an inhibitor of viral RNA synthesis[3]. Sofosbuvir has a variety of adverse effects including fatigue, headache, nausea, rash and irritability[4], other adverse side effects were recorded as insomnia and asthenia [5]. However, the most commonly reported side effects are brain fog (confusion, memory loss and sudden blankness), depression and mood swings[6]. Brain fog may be caused by cognitive impairments[7] as a result of chronic hypoperfusion and the oxidative stress,

progressive cognitive deficit indicates the progress of neuronal damage [8], [9].

2. Methodology:

Animals

Twenty adult male albino rats were used in this experiment; each of them is weighing 150-200 grams. Food and water were provided *ad libitum* for 7 days before use in the anatomy department, faculty of medicine, Menoufia University. All aspects of animal care and treatment were carried out according to the local guidelines of the ethical committee for animal research.

Experimental design

The animals were divided into two groups:

Group I (Control): included ten rats were kept without any treatment throughout the experimental period.

Group II (Sovaldi treated): included ten rats that received Sofosbuvir, a product of Pharco Pharmaceuticals, Alexandria, Egypt, was available in the form of tablets with the trade name Gratisovir. Each tablet contained 400 mg of Sofosbuvir; these tablets were dissolved in distilled water and given for rats orally by gastric tube in a dose of 4mg/kg per day for 5 weeks.

Histological study**Light microscope**

Brain samples were fixed in 10% formol saline and processed to prepare 5 μm -thick paraffin sections for:

- Haematoxylin and Eosin (H&E) stain.
- Immunohistochemical stains for brain sections.

(1) Polyclonal rabbit anti-Ki67 antibody (Labvision Corporation; ThermoScientific, USA). Working dilution 1:500.

(2) Polyclonal goat anti-GFAP antibody (Labvision Corporation; ThermoScientific, USA). Working dilution 1:300.

Electron microscope

Small pieces (1 mm^3) from the visual area were rapidly cut and immediately fixed in 0.1 mol/l phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde at 4°C overnight in phosphate buffer in the refrigerator. Specimens were then fixed in 1% osmium tetroxide for 1h at 4°C. After repeated washing, specimens were dehydrated in graded ethanol and embedded in Epon. Semithin sections (1 μm) were cut by ultramicrotome (Leica

ultracut UCT, Germany) using glass knife, stained with 1% Toluidine blue and examined with light microscope to select areas for subsequent ultramicrotomy. Ultrathin sections (60–80 nm) were cut using diamond knife and double stained with uranyl acetate and lead citrate [10]. Sections were examined and photographed with transmission electron microscope (JEOL 100 CX, Japan), Faculty of Science, Menoufia University.

Morphometric and statistical analysis

The data was obtained using Leica Qwin 500 Image analyzer computer system (England). We measured the area percent for Ki67 and GFAP immunoreaction in astrocytes and its processes in visual cerebral cortex using magnification 400 with a measuring frame area 7286.78 μm^2 . Statistical analysis was carried out on all parameters. The data obtained were expressed as mean values \pm SD and analyzed using an unpaired student's *t*-test. Differences were considered to be significant at *P* value less than 0.05 against the control group.

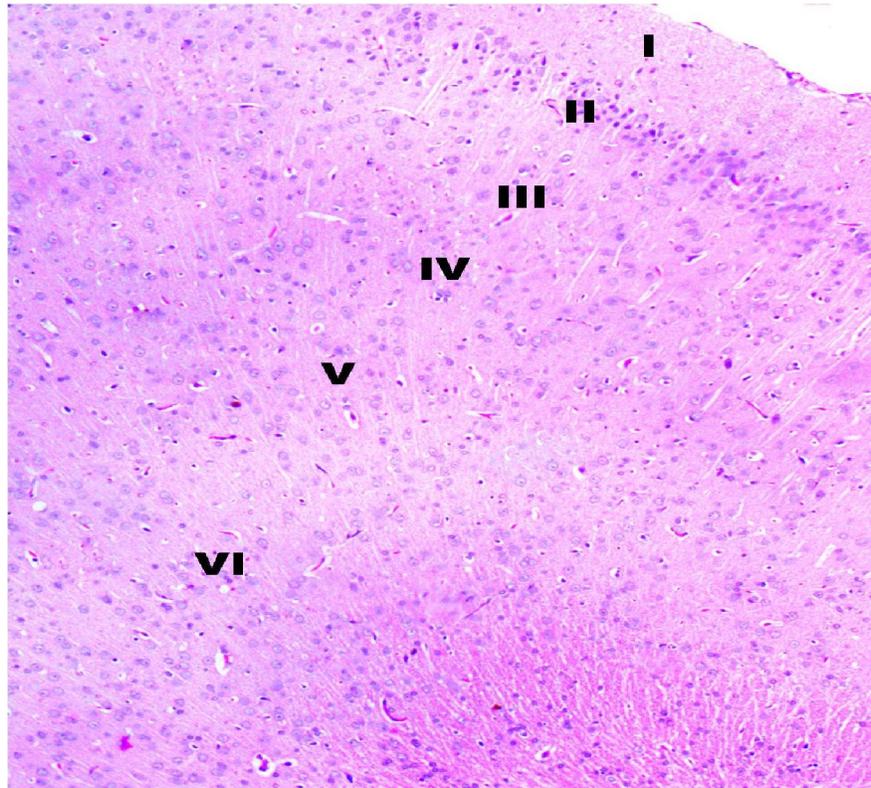
3. Results:

Figure 1: A photomicrograph of a section in the visual cerebral cortex of control rat showing normal appearance of the six layers: outer molecular layer (I), external granular layer (II), external pyramidal layer (III), internal granular layer (IV), internal pyramidal layer (V), and polymorphic layer (VI). H&E, $\times 100$.

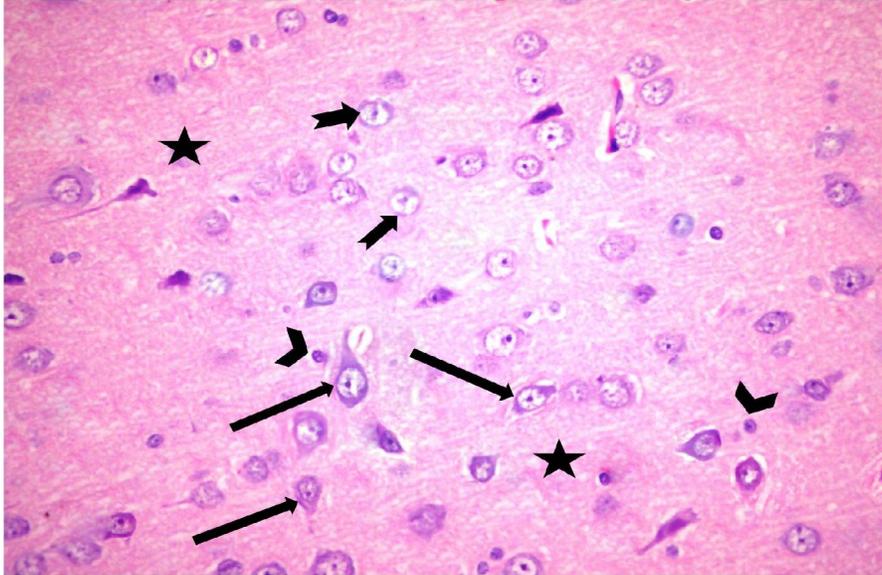


Figure 2: A photomicrograph of a section in the internal granular and internal pyramidal layers of cerebral cortex of a control rat showing pyramidal cells with open face rounded nuclei, basophilic cytoplasm, and long apical dendrites (arrows). The granular cells (notched arrows) show large open face nucleus with prominent nucleolus and little cytoplasm. Perineural neuroglia cells (arrow head) and blood capillaries (V) are seen. The pink-stained background, the neuropil (asterisks) is a mat of neuronal and Glial cell processes. H&E, $\times 400$.

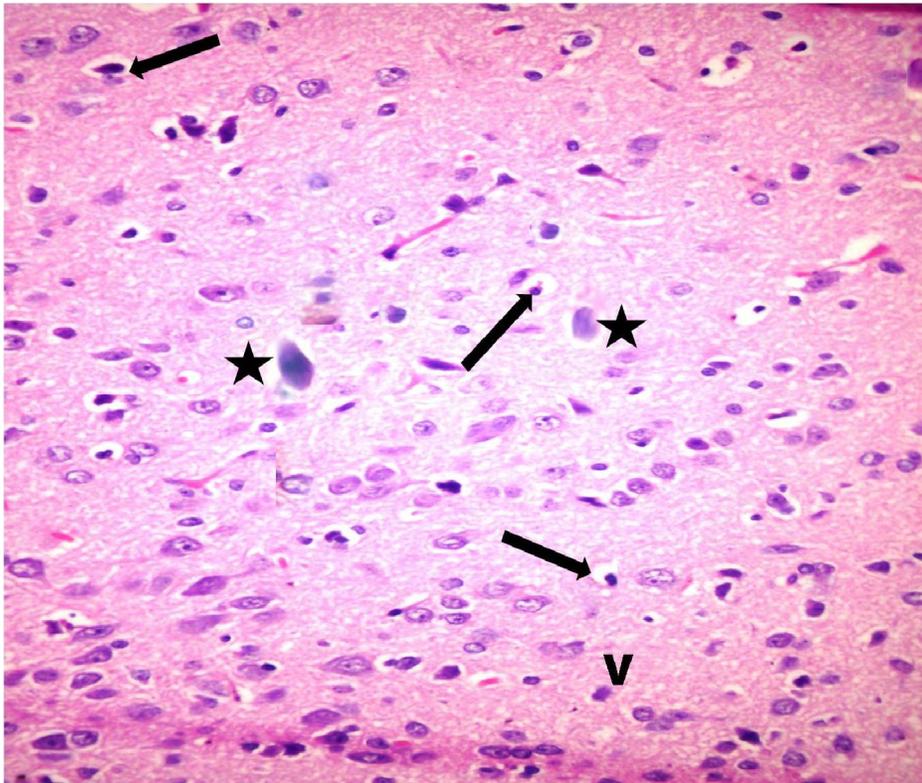


Figure 3: A photomicrograph of a section in the internal granular and internal pyramidal layers of cerebral cortex of Sovalditreated rat showing hypercellularity of the cortex. Shrunken cells with pyknotic nuclei (arrows) are clearly seen. Some cells appear as ghosts (deeply stained nuclei surrounded by clear halos) (asterisks). H&E, $\times 400$.

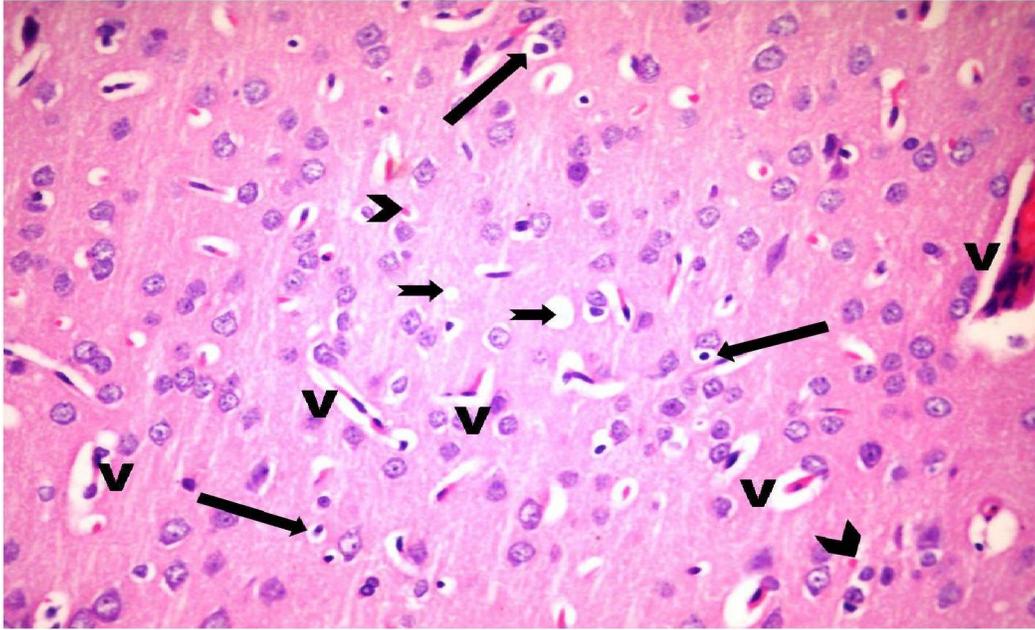


Figure 4: A photomicrograph of a section in the internal granular and internal pyramidal layers of cerebral cortex of sovaldi treated rat showing many shrunken cells with pyknotic nuclei (arrows). Dilated congested blood vessels (V) and extravasation of blood (arrow head) are clearly seen. Note also the vacuolated neuropil (notched arrow). H&E, $\times 400$.

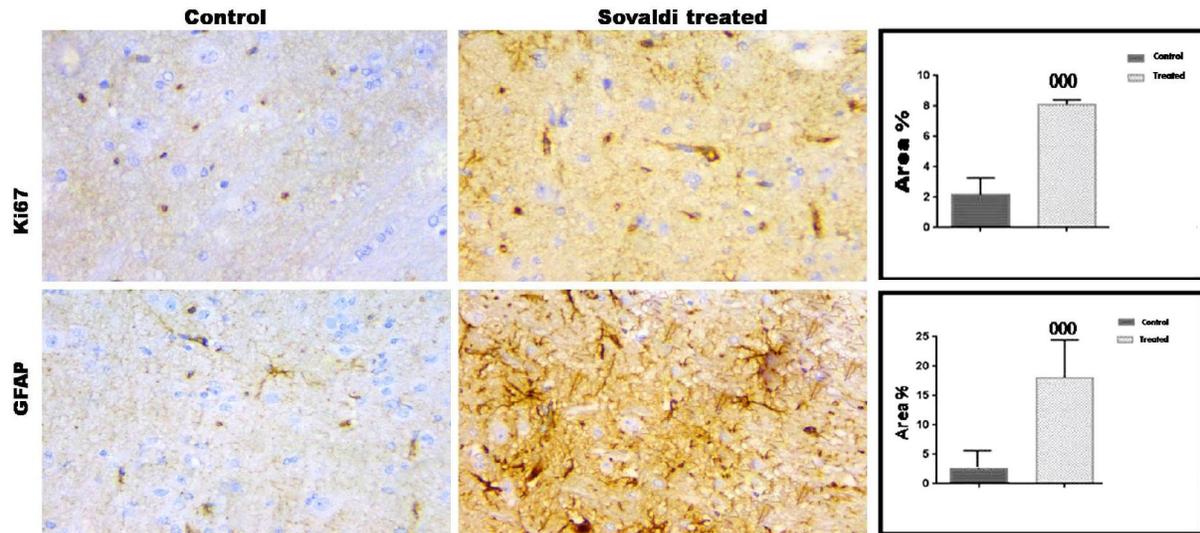


Figure 5: Representative immunohistochemical staining of cortical sections of both experimental groups. Sovaldi treated sections showed a significant up-regulation in area % of both Ki67 and GFAP immunostained cells as compared to control group. $^{000}p < 0.001$, compared to the control group. Immunoperoxidase technique, $\times 400$.

H&E-stained sections of control group revealed that six layers forming the cortical area could be identified: I, the outer molecular layer; II, the external granular layer; III, the external pyramidal layer; IV, the inner granular layer; V, the inner pyramidal layer; and VI, the polymorphic layer (Fig. 1). The pyramidal cells, granular cells, and perineural neuroglia represented the main cellular components of the

cortical area. These cells were scattered in an eosinophilic background (neuropil) formed of neuronal and glial cell processes. Pyramidal cells showed vesicular nuclei, basophilic cytoplasm, and long apical dendrites. Granular cells appeared rounded in shape and showed larger rounded open face vesicular nuclei with prominent nucleoli. Glial cells appeared

smaller in size with small deeply stained nuclei (**Fig. 2**).

Examination of H&E stained sections of the visual cortex of Sovaldi treated rats showed hypercellularity. Most neurons were distorted in shape and shrunken with deeply stained nuclei and surrounded by vacuolated pale areas most probably apoptotic cells. Ghost masses with deeply stained fragmented nuclei and surrounded by clear halos were detected (**Fig 3**). Neuropil appeared vacuolated and showed vascular dilatation and extravasation of blood (**Fig 4**). Some cells appeared normal.

Sovaldi treatment dramatically up-regulated the expression of Ki67 of brain sections (Area % 78.45 ± 2.76 Vs 14.23 ± 3.49). In addition, expression of GFAP was also significantly enhanced in brains of the Sovalditreated group (Area % 69.35 ± 1.39 Vs 19.73 ± 2.52) (**Fig 5**).

Examination of ultrathin sections of the control visual cortex revealed that pyramidal and granular

cells of internal pyramidal layer had large euchromatic nuclei. Their cytoplasm contained mitochondria, rough endoplasmic reticulum and granules. The surrounding neuropil contained some myelinated and unmyelinated nerve fibers (**Figs 6 and 7**). The myelinated axons (axons) in the neuropil showed regular arrangement of the myelin lamellae, and the axoplasm contained mitochondria and neurofibrils (microtubules and microfilaments) (**Fig. 8**).

Electron microscopic examination of Sovaldi treated group showed that some pyramidal and granular neurons exhibited irregular heterochromatic shrunken nuclei and peripheral clumping of chromatin. Cytoplasm appeared rarefied and showed degeneration of cellular organelles with cristolysis of the mitochondria (**Figs. 9 and 10**). The axons exhibited irregular contour and multiple splitting of their myelin lamellae with wide spaces in between. Axoplasmic vacuoles were also observed (**Fig. 11**).

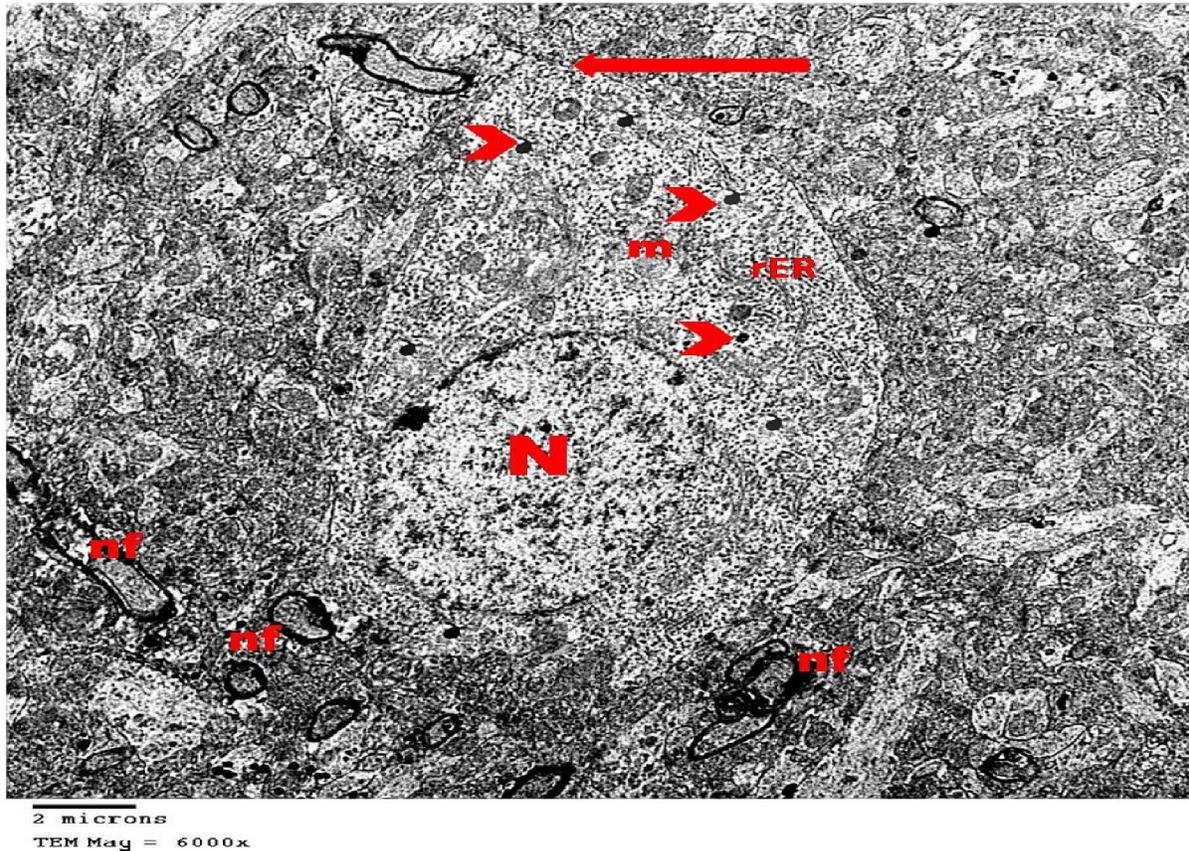
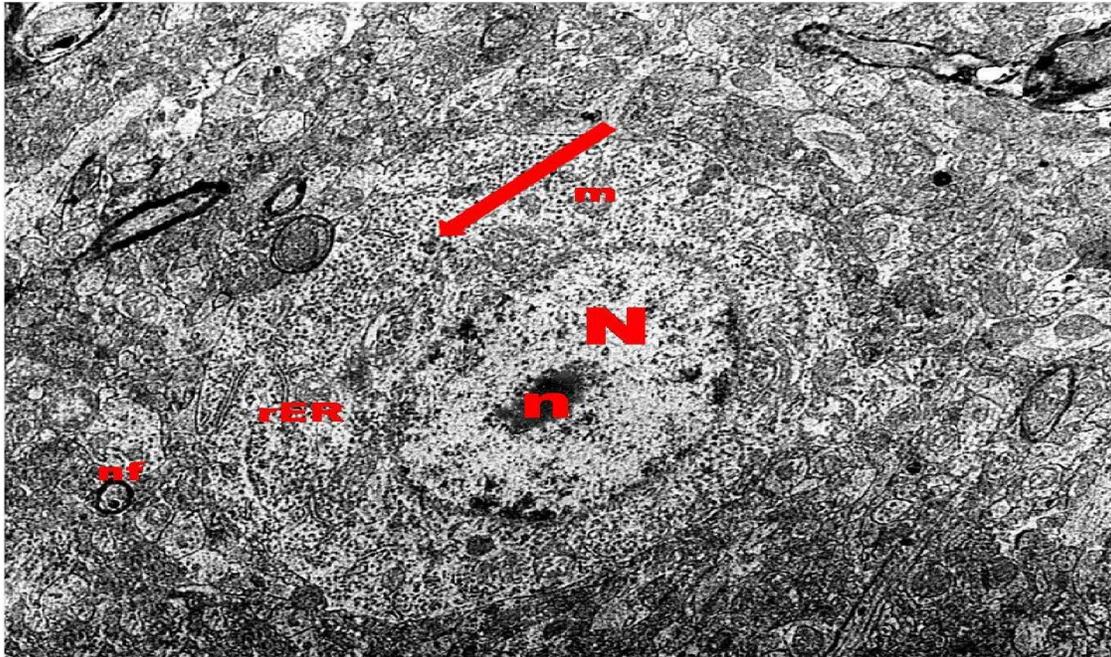
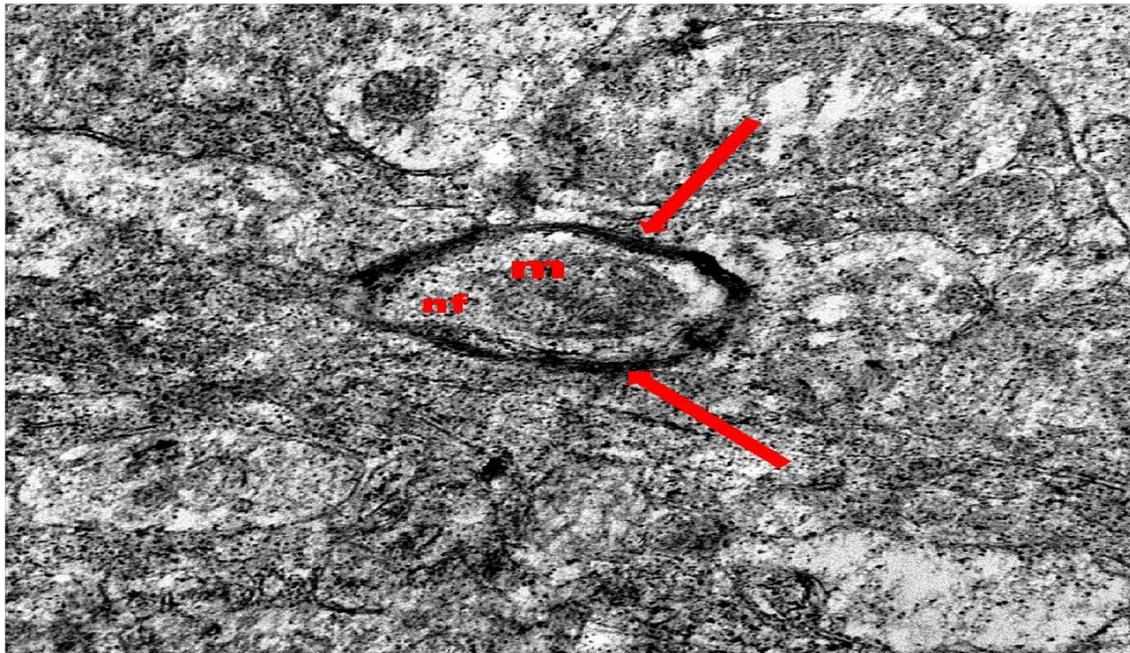


Figure 6: An electron micrograph of internal pyramidal layer of a control rat showing a pyramidal neuron with large rounded euchromatic nucleus (N) and long apical dendrite (arrow). Its cytoplasm contains mitochondria (m), rough endoplasmic reticulum (rER) and Nissl's granules (arrow heads). The surrounding neuropil appears with nerve fibers (nf). TEM×6 000.



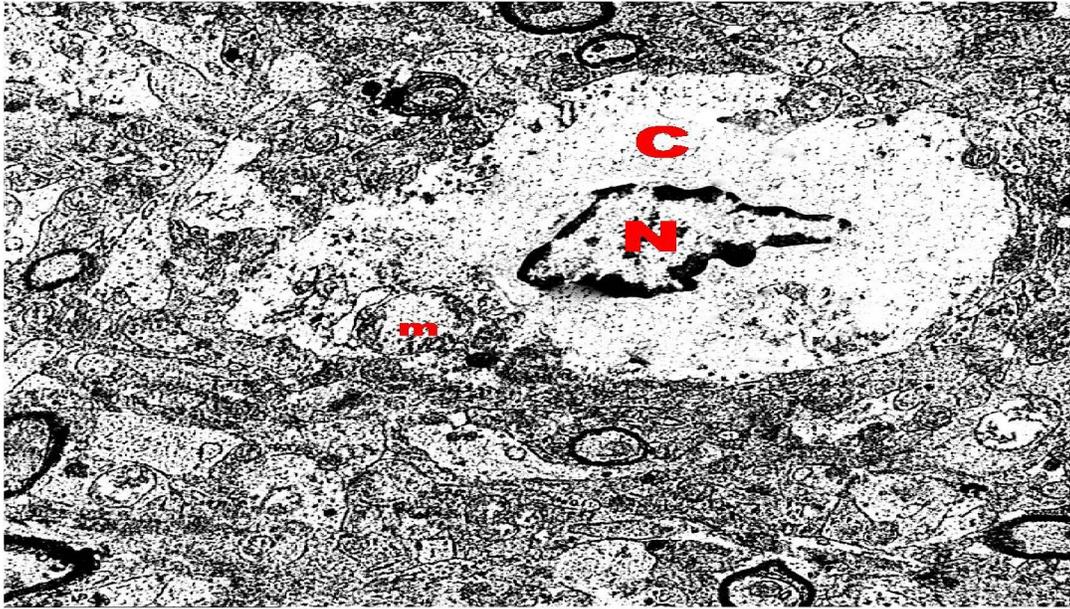
2 microns
TEM Mag = 8000x

Figure 7: An electron micrograph of internal pyramidal layer of a control rat showing a granular neuron with large open face nucleus (N), prominent nucleolus (n) and little cytoplasm. Its cytoplasm contains mitochondria (m), rough endoplasmic reticulum (rER) and Nissl's granules (arrow). The surrounding neuropil appears with nerve fibers (nf). TEM×8 000.



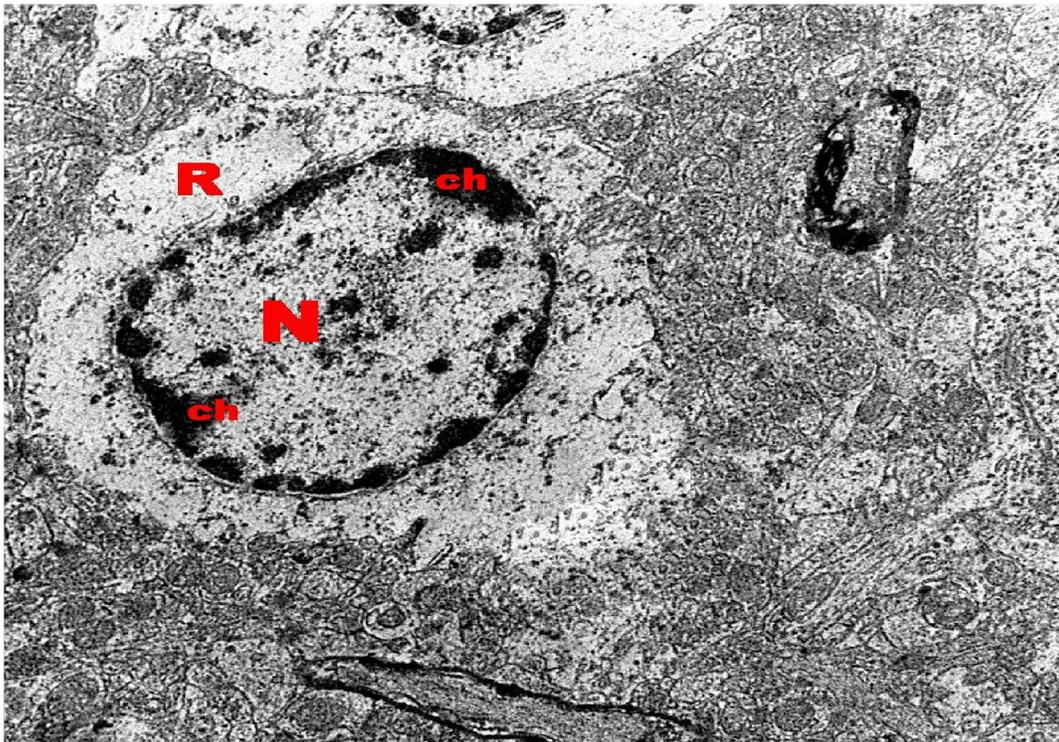
500 nm
TEM Mag = 40000x

Figure 8: An electron micrograph of internal pyramidal layer of a control rat showing a myelinated axon in the neuropil with myelin lamellae (arrows). The axoplasm contains mitochondria (m) and neurofibrils (nf). TEM×40 000.



2 microns
TEM Mag = 12000x

Figure 9: An electron micrograph of internal pyramidal layer of Sovaldi treated rat showing a pyramidal neuron with shrunken irregular shaped nucleus (N). The cytoplasm (C) showing destroyed organelles and few swollen mitochondria with cristolysis (m). TEM×12 000.



2 microns
TEM Mag = 12000x

Figure 10: An electron micrograph of internal pyramidal layer of Sovaldi treated rat showing a granular neuron with irregular nucleus (N) and peripheral clumping of chromatin (ch). The cytoplasm appeared rarefied (R) with severely destroyed organelles. TEM×12 000.

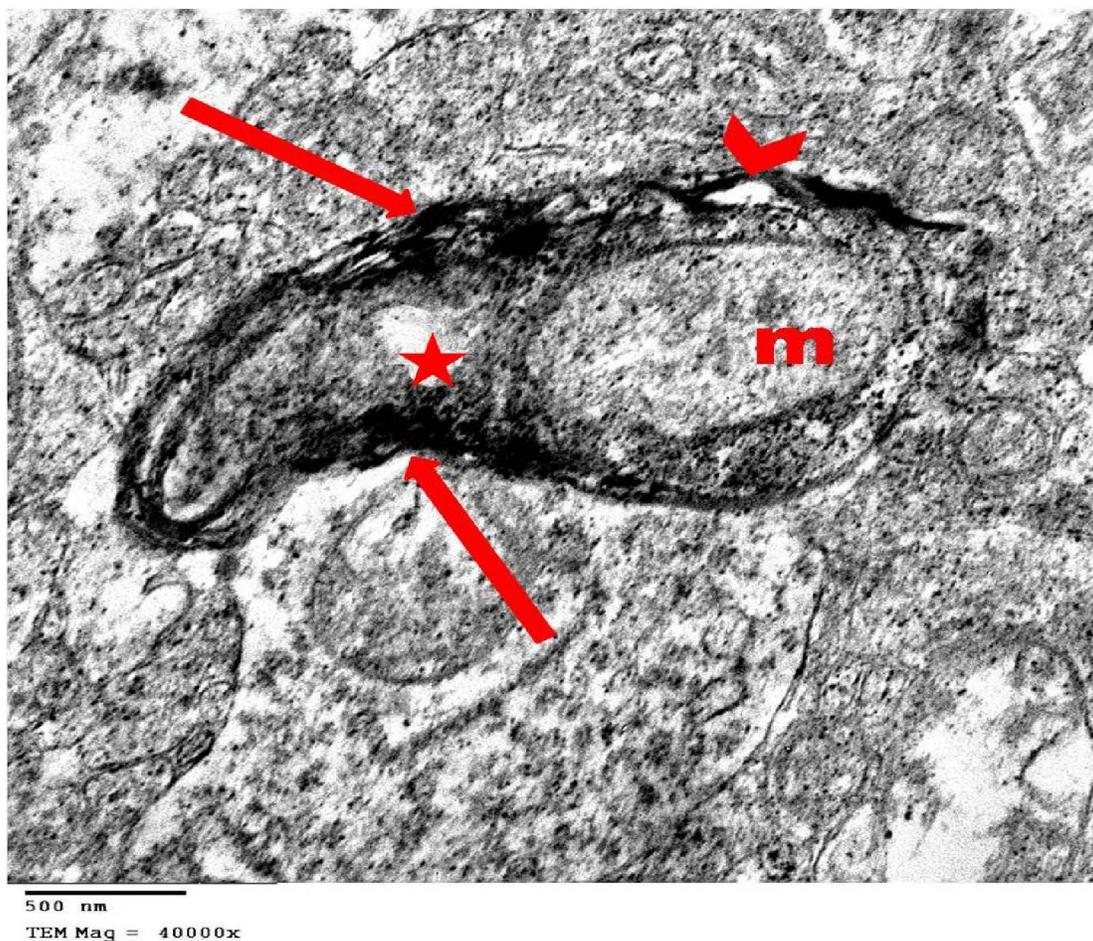


Figure 11: An electron micrograph of internal pyramidal layer of Sovaldi treated rat showing myelinated axon in the neuropil with irregular contour myelin lamellae (arrows) and focal loss of the myelin lamellae (arrow head). The axoplasm contains swollen mitochondria with destructed cristae (m) and axoplasmic vacuole (star). TEM \times 40 000.

4. Discussion:

Sofosbuvir induced brain fog as well as blurred vision necessitated the aim of this study which was the demonstration of the histological adverse effects of the sofosbuvir administration on the visual cortex, examination of visual cerebral cortex slides of sofosbuvir treated rats showed hypercellularity, distorted neuronal shape, appearance of apoptotic cells as well as ghost masses, vacuolated neuropil which showed vascular dilatation and extravasation of blood, these changes were described by other authors as neurotoxicity and encephalopathy which might cause impairment or loss of the function [11]. The distorted neurons were previously explained by the impaired biosynthesis of cell proteins; nucleic acids, certain enzymes and various neurotransmitters [12]. This impairment might cause decreased levels of myelin basic protein leading to degeneration of myelin sheath and nerve fibers and so decreased cognitive scores together with aging [13]. This was in agreement with our TEM findings of sofosbuvir treated cerebral cortex

which included focal loss of the myelin lamellae of the neuronal axons and its axoplasm appeared vacuolated containing swollen mitochondria with destructed cristae.

The neuropil vacuoles were considered as pericellular spaces that resulted from the shrinkage of cells and withdrawal of their processes secondary to cytoskeletal affection. So this emphasized on the neuronal death and necrosis [14]. This death might be due to the oxidative stress as postulated by some authors [15]. This explained our TEM results which included heterochromatic shrunken nuclei with peripheral clumping of chromatin; cytoplasmic rarefaction and degenerated cellular organelles with cristolysis of the mitochondria. Concurrent cerebral necrosis with congested and dilated blood vessels were attributed to inflammation of the blood vessels wall which led to hypoperfusion and necrosis [16]. In our study, normal cerebral cortex slides stained with ki67 showed strong expression to this proliferative marker which indicated the presence

of the quiescent progenitor cells, this expression was increased in the slides of the group treated with sofosbuvir, this was in agreement with other authors who stated that ischemic brain injury stimulated the proliferation of neural progenitor cells to restore populations that were lost by injury [17]. This also explained the hypercellularity of Sofosbuvir treated cerebral cortex. The up regulation of GFAP marker was considered by other authors as indicator to the active astrogliosis, which was a sign of inflammation and aging in the brain [18], this explained the increased expression of the GFAP in the cerebral cortex slides of sofosbuvir treated rats.

Conclusion:

We concluded that the use of sofosbuvir was accompanied by neurotoxicity and degeneration of the neuronal axons, apoptosis of the pyramidal cells, inflammation of cerebral vessels causing ischemic necrosis of the visual cerebral cortex and aging. All these findings might lead to blurring of vision.

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