The possible protective role of vitamin C on rat parotid gland exposed to mobile phone radiation

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SUMMARY

The widespread use of cell phones recently has raised interest in studying the safety of exposure to their electromagnetic waves. This study aimed to evaluate the possible protective role of vitamin C (Vit. C) against the pathological effects produced by the relatively long duration of third-generation mobile phone radiation exposure in the parotid gland of male white albino rats. Also, to detect the expected recovery after 4 weeks of withdrawal by immunohistochemical, histopathological, and biomolecular studies. Forty male adult white albino rats were used in this study, equally divided into 4 groups of 10 rats in each group: Group I served as the sham control group; Group II was exposed to radiation by phone generator (2100 MHz) for 3 hours/day for 5 days/week for 6 weeks; and Group III was exposed to phone radiation similar to Group II and supplemented with Vit. C at a dose of 40 mg/kg for 5 days per week for 6 weeks. Group IV (withdrawal) was exposed to radiation as Group II, then left without intervention for 4 weeks. Histological and immunohistochemical, and the biochemical evaluation of oxidative stress within parotid tissue (malonaldehyde (MDA), superoxide dismutase (SOD), salivary total protein, and amylase activity). qRT-PCR for TGF, MMP2, NFKB, and TNF gene expression was performed in this study. Histological examination of the parotid gland in group II showed many histological changes, such as degenerative changes in the duct system, epithelial lining of the acini, interstitial space which different collagen fibers and polysaccharides, blood capillaries, and nuclei. Also, alpha smooth muscle actin (α-SMA) and Ki-67 were aggravated. The statistical analysis showed that treating rats with vitamin C improved the parotid glands' ability to secrete amylase activity and total protein. It also fixed the antioxidant. Inflammatory cytokines TNF, NFKB, and fibrotic marker TGF and MMP2 gene expression were significantly upregulated in the exposed group,

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which was restored nearly to the control group after Vit C supplementation. Long-term exposure to mobile radiation affected the secretory function and the histological structure of the parotid tissues. Vitamin C supplementation ameliorated these pathological effects.

Key words: Mobile phone radiation – MDA – SOD – Vitamin C – α-SMA

INTRODUCTION

In recent times, non-ionizing radiation, particularly microwave radiation emitted by mobile phones, has become ubiquitous, with almost the entire global population utilizing these devices (Rosado et al., 2014). The potential health impacts of mobile phone radiation had been the previously studied with relevant positive association. The International Agency for Research on Cancer (IARC) has classified the electromagnetic fields (EMF) generated by mobile phones as potentially carcinogenic, emphasizing the importance of ongoing public health surveillance. Over the last two decades, several studies had been developed on the effects of mobile phone radiation on head and neck cancers, with the parotid gland being identified as particularly vulnerable due to its proximity to mobile phone usage. The widespread adoption of 3G mobile technology has raised concerns about both the thermal and non-thermal effects of electromagnetic radiation. There is growing apprehension regarding the mechanisms by which mobile phones may exert their adverse effects, including the potential for inducing oxidative stress and disrupting antioxidant defense systems. Saliva, a critical component in maintaining oral health, is produced predominantly by the major salivary glands, with the parotid gland contributing significantly. Disruptions in salivary gland function can lead to xerostomia, a condition with challenging side effects, highlighting the urgency for effective protective measures against mobile phone radiation. This study aims to explore the protective effects of Vitamin C on the parotid glands of male Albino rats exposed to 3G mobile phone radiation, assessing both the immediate and long-term recovery potential through histopathological, immunohistochemical, and biomolecular analyses.

MATERIALS AND METHODS

Used drug

Effervescent Vit C (vitacid C) tablets were purchased from Chemical Industries Development Company, Giza, Egypt. Each tablet contains 1000 mg of Vit C.

Animals

The study included 40 healthy adult male white albino rats had 3-4 months age, weighing 200-230 gm obtained from the animal house of Faculty of Medicine Helwan University and handled according to the guidelines after the approval of the Research Ethics Committee (REC) for human and animal research with approval number (REC-FM-HU-38-2023) from the animal house-Faculty of Medicine Helwan University. The animals were maintained at 50-70% humidity, temperature of 22 ± 2°C and with a 12 h light/dark cycle. All efforts were done to reduce animals' suffering. Sample size was calculated using the G power software. Based on Rosado et al. (2014), we found that ten rats per group were an appropriate sample size for the study, with total sample size 40 rats (four groups). α error probability = 0.05, power = 80% and, effect size = 0.56, after acclimatization, rats were randomly divided equally into 4 groups (10 rats for each group):

- Group I (Sham control): rats were given 0.5 ml of distilled water once /day/orally for 5 days/ week for 6 weeks.
- Group II (irradiated group): rats were exposed to mobile phone generator radiation 2100 MHz EMF 3hrs/day (between 10 am and 1 pm) for 5 consecutive days /week for 6 weeks.
- Group III:(irradiated + vitamin C group): rats were given 40mg/kg Vit C orally, one hour before exposure to irradiation as in group II (Jelodar et al., 2013).
- Group IV: (irradiated + withdrawal group): rats were exposed to irradiation as in group II, then left for withdrawal without intervention for further4 weeks.

Exposure system and application of electromagnetic field

Animals were exposed to 2100 MHz radiofrequency radiation emitted by an electromagnetic field generator (Trannsteltrafo. LTS 602) simulating a 3G mobile phone for 3hrs/day (between 10 am and 1 pm) for 5 consecutive days /week for 6weeks. A specially designated exposure system of electromagnetic field (EMF) was used with dipole exposure antenna and round plastic tube cage (Holiday Industries Inc., UK) (El-Bediwi et al., 2011). The animals were sacrificed by cervical dislocation (El-Akabawy et al., 2015) at the end of the experiment, right parotid glands of all rats were carefully dissected and divided into two parts. One part was used for histological evaluation, and the other is for biomolecular analysis.

Biomolecular analysis

Estimation of oxidative markers MDA and antioxidant marker SOD

Parotid tissue Malondialdehyde (MDA) was assessed by ELISA technique according to kit instruction: MYBio source, USA. Cat # MBS2605193 Parotid tissue superoxide dismutase (SOD) was assessed by ELISA technique according to kit instruction: MYBio source, USA. Cat # MBS036924.

Collection and preparation of saliva

Whole saliva was collected after 10-hrs fasting according to a standardized method. Using 0.1 ml/kg Hypnorm IM (Janssen Pharmaceutical, Beerse, Belgium) The animals were anaesthetized. Saliva secretion was stimulated by injection of a combination of 2.5 mg/kg body weight of isoproterenol (Sigma Chemical Co., St. Louis, MO) and 2.5 mg/kg body weight of pilocarpine (Sigma Chemical Co., St. Louis, MO) subcutaneously. To prevent the contamination from tears and nasal secretion, the rats were put with their mouth placed over a plastic cup; also to ensure a constant body temperature for 3-5 minutes during saliva collection, the rats were placed under electric lamps. The collected saliva samples were centrifuged for 10 minutes at 3000 rpm; the biochemical parameters of the supernatants were analyzed (Johansson et al., 1989).

Estimation of salivary amylase activity

Salivary amylase activity was assessed by ELISA technique according to kit instruction: MYBio source, USA. Cat # MBS8806064.

Estimation of salivary total protein

Salivary total protein was assessed by ELISA technique according to kit instruction: MYBio source, USA. Cat # MBS3808613.

Quantitative RT-PCR for studied genes

Parotid tissues from all groups were homogenized for total RNA extraction in 300 µl of lysis buffer according to kit instruction Gene JET Kit (Thermo Fisher Scientific Inc., Germany, #K0732). 5 µl from the total RNA were used for qRT-PCR using the Step-one instrument (Applied Biosystems, U.S.A.) according to kit instructions (SensiFASTTM SYBR R Hi-ROX) (catalog number PI-50217 V). Thermal cycles were as follows: 15min for 45°C for one cycle for cDNA synthesis, 10 min at 95°C for reverse transcriptase enzyme inactivation, followed by 40 cycles for amplification. Each cycle was continued for: 10 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The expression of studied genes was normalized to GAPDH and analyzed by ΔΔ ct method.

Primer sequences for TGF gene:

forward, 5′-CCCAGCATCTGCAAAGCTC3′

reverse, 5′GTCAATGTACAGCTGCCGCA3′

MMP2gene

Forward, 5′GTCTTCCCCTTCACTTTTCTG3′

reverse, 5′CGGAA GTTCTTGGTGTAGGTG3′

NFKB gene

forward, 5′AATTGCCCCGGCAT3′

reverse, 5′TCCCGTAACCGCGTA3

TNF gene

forward, 5′CTCTTCTGCCTGCTGCACTTTG3′

reverse, 5′ATGGGCTACAGGCTTGTCACTC3′

GAPDH gene

forward, 5′CATGTTCGTCATGGGGTGAACCA3′

reverse, 5′AGTGATGGCATGGACTGTGGTCAT-3′

Histological study

The right parotid salivary glands of all rats were dissected and were fixed in neutral buffered 10% formalin, dehydrated, embedded in paraffin, sections of 5 µm-thick were cut, stained with H&E to analyze the histological structure, Masson's trichrome stain was done to assess collagen fiber content. The presence of glycoprotein at the basement membrane was detected by Periodic acid Schiff (PAS) staining histochemical study (Suvarna et al., 2013).

Immunohistochemical study

In this study using immunohistochemistry for alpha smooth muscle actin (αSMA) helped identify the myoepithelial cells (MECs) during their differentiation **(**Suvarna et al., 2013), and Ki- 67 immune staining was used to detect the cellular proliferation (Maher et al., 2020).

Immunostaining for α-SMA

It was performed on deparaffinized, rehydrated, sections of 5-µm-thick were blocked with 1.5% normal goat serum in PBS. The sections were incubated with 6 ml prediluted primary (1ry) mouse monoclonal antiα-SMA antibody (Ab) (ab5694) (Dako Corporation, Glostrup, Denmark) for 45 minutes at room temperature, then incubated with a second-stage biotinylated antibody for 1 h, at room temperature (biotin conjugated goat anti-rabbit IgG, 1:200,). The reaction products were visualized after rinsing in PBS by immersing the section into the chromogen diaminobenzidine. Finally, counterstaining of the sections was done with Mayer's hematoxylin, dehydrated, and mounted. The positive cells for α-SMA showed brown cytoplasmic reaction. The negative control was processed in the same way, but omitting the step of 1ry Ab (Suvarna et al., 2013).

Immunostaining for Ki-67

5-µm paraffin sections were used for immunohistochemical detection of Ki-67 antigen using a rabbit monoclonal antibody. Hydration, and blocking of endogenous peroxidase was done after standard deparaffinization, sections were processed in a microwave oven twice (5 min each) at high power. Followed by a standard streptavidin-biotin peroxidase technique to detect the antigen. Sections were incubated at room temperature with anti-Ki-67 (dilution 1:40, DAKO, USA) for 30 min diaminobenzidine (Sigma Fast DAB). Mayer's haematoxylin solution was used for counterstaining. The same streptavidin–biotin technique was used in tissue sections, where 1% BSA in PBS replaced the primary antibody for negative control. The nuclear (nucleoplasmic) staining for Ki-67 was considered positive (Maher et al., 2020).

Morphometric study

Using Leica Quin 500LTD (Cambridge, UK) computer assisted image analysis system. The count of vesicular nuclei was performed in H&E-stained sections. The area percentage of collagen fibers stained with Masson trichrome and PAS positive staining of basement membrane was measured using binary mode.

The area percentage of α-SMA +ve MECs and that of Ki-67 positive nuclei were performed using interactive measurements menu (The Ki-67-positive nuclei were counted through a minimum of 200 cells per field in five different fields at ×400 magnification). The Ki-67 labeling index (Ki-67 LI) is the number (%) of positive cells (Luo et al., 2005; Hashemipour et al., 2014). The measurements were done in 10 high power fields.

Statistical analysis

Statistical analysis of the studied parameters was performed by using SPSS program (version 25; SPSS Inc., Chicago, Illinois, USA). Values were expressed as means ± SD and compared using ANOVA test. $P < 0.05$ was considered statistically significant (Emsley et al., 2010).

RESULTS

General observations

No rat deaths were observed during the experiment. The electromagnetic fields were well tolerated by the animals throughout the study.

Histological results

Haematoxylin.and.Eosin.stain

G*roup I (sham control group)*: The sections of parotid glands of the control group using the light

microscope showed the normal architecture. It is formed of stroma, which consists of the connective tissue capsule and the septa extending from capsule and dividing the glands into lobules. The parenchyma between the septa consists of groups of pure serous acini, and were lined by pyramidal cells having rounded central vesicular nuclei and basal basophilic cytoplasm. The duct portion, consisting of striated, interlobular and intra-lobular ducts, were easily detected. There was a network of capillaries in between the acini and in a connective tissue septum (Fig. 1A).

G*roup II* showed widened connective tissue septa between some lobules and homogenous acidophilic material in others; besides, disorganized acini in some areas could be observed. Some acinar cells showed dark nuclei and others showed cytoplasmic vacuoles. Inter-lobular septa contain dense connective tissue fibers with cellular infiltration between the lobules and around a dilated duct. Dilated and congested blood vessels were seen. There was also loss of the architecture of multiple lobules (Fig. 1B).

G*roup III (irradiated + Vit C)* showed the capsule, septa between the lobules. Also, an inter-lobular duct in a connective tissue septum and intra-lobular ducts in between the acini apparently normal serous acini, intra-lobular duct, interlobular ducts and non-congested blood vessel in a slightly thickened connective tissue septum containing homogeneous material (Fig. 1C).

G*roup IV (irradiated + Withdrew)*: Apart from the presence of few vacuolations, few pyknotic nuclei, and slightly congested blood vessels, it showed apparently normal histological features (Fig. 1D).

Masson's Trichrome Stain

Group I (sham control group): few blue collagen fibers in the capsule, fibers in the septa between the lobes and the lobules and around the interlobular ducts and blood vessels were detected (Fig. 2A).

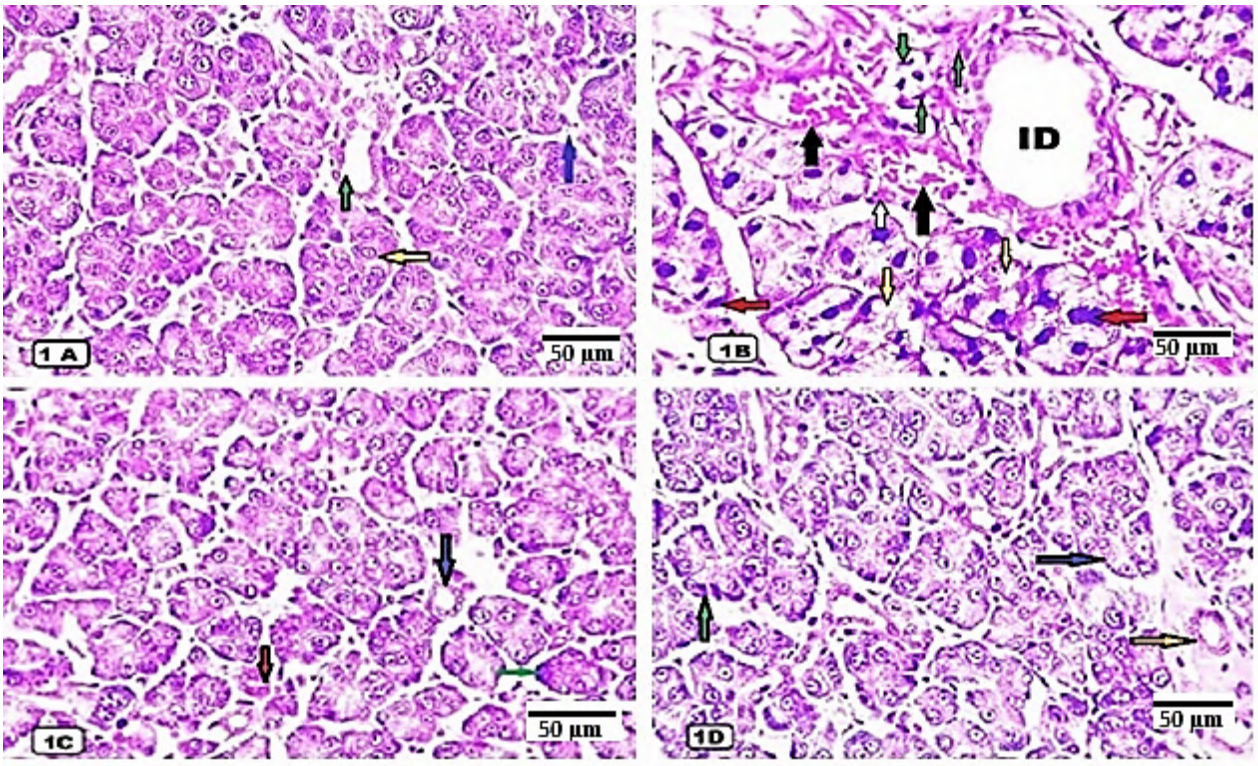

Fig. 1.- H&E-stained parotid gland sections of all groups. x 400. Scale bars = 50 µm. (**1A**): Group I (sham control) showing rounded serous acini (yellow arrow) with narrow lumen. They have basal vesicular nuclei and basal basophilia. Note normal appearance of intralobular ducts (green arrow) and thin septa (blue arrow). (**1B**): group II (Radiation) showing irregular acini with ill-defined outline and darkly stained nuclei (red arrows) and cytoplasmic vacuoles (yellow arrows) and homogenous acidophilic material (white arrows). Cellular infiltration (green arrow) inside thick connective tissue septa and around a dilated interlobular duct (ID) with dilated congested blood vessels (black arrows). (**1C**): group III (Radiation +Vit C) showing acini and interlobular ducts nearly as control group (blue arrow) with few dark stained nuclei (green arrow), septa are also seen showed nearly normal blood vessels (red arrows). (**1D**): Group IV (Radiation + Withdrawal) showing nearly as control in acini (blue arrow) and intralobular ducts (yellow arrow) but few of dark stained nuclei and some vacuoles in few acini could be detected (green arrow).

G*roup II (irradiated)*: Multiple blue collagen fibers in the capsule, excessive collagen fibers in interlobular septa, around interlobular ducts in addition to blood vessels were detected (Fig. 2B).

G*roup III (irradiated+ Vit C)*: Sections clarified that the low amount of collagen fibers appeared in between the lobes and lobules and around ducts and blood vessels (Fig. 2C).

G*roup IV (irradiated +Withdrew)*: sections clarified that a moderate amount of collagen fibers (more than in Group III) appeared in between the lobes and lobules and around ducts and blood vessels (Fig. 2D).

PAS reaction

Group I (sham control group) showed obvious positive (a magenta color) PAS reaction in the basement membrane of the acini (Fig. 3A).

G*roup II (irradiated)*: The basement membrane of some acini showed moderate positive PAS reaction and absent in others (Fig. 3B).

G*roup III (irradiated + Vit C)* showed obvious positive PAS reaction in the basement membrane of the acini (Fig. 3C).

G*roup IV (irradiated + Withdrew)* showed obvious positive PAS reaction in the basement membrane of the acini nearly as in Group III (Fig. 3D).

Immunohistochemistry

Alpha smooth muscle actin (α-SMA)

Group I (sham control group): Immunoreaction to α-SMA showed brown cytoplasm in multiple MECs at the periphery of the acini and intra-lobular ducts. Mild positive reaction is also seen in the lining of blood vessels (Fig. 4A).

G*roup II (irradiated group)*: Section showed an increase in α-SMA immunoreaction at the interlobular ducts and periphery of the acini (Fig. 4B).

G*roup III (irradiated + Vit C group)*: Immunoreaction to α-SMA Sections showed α-SMA immunoreaction in multiple MECs at the periphery of

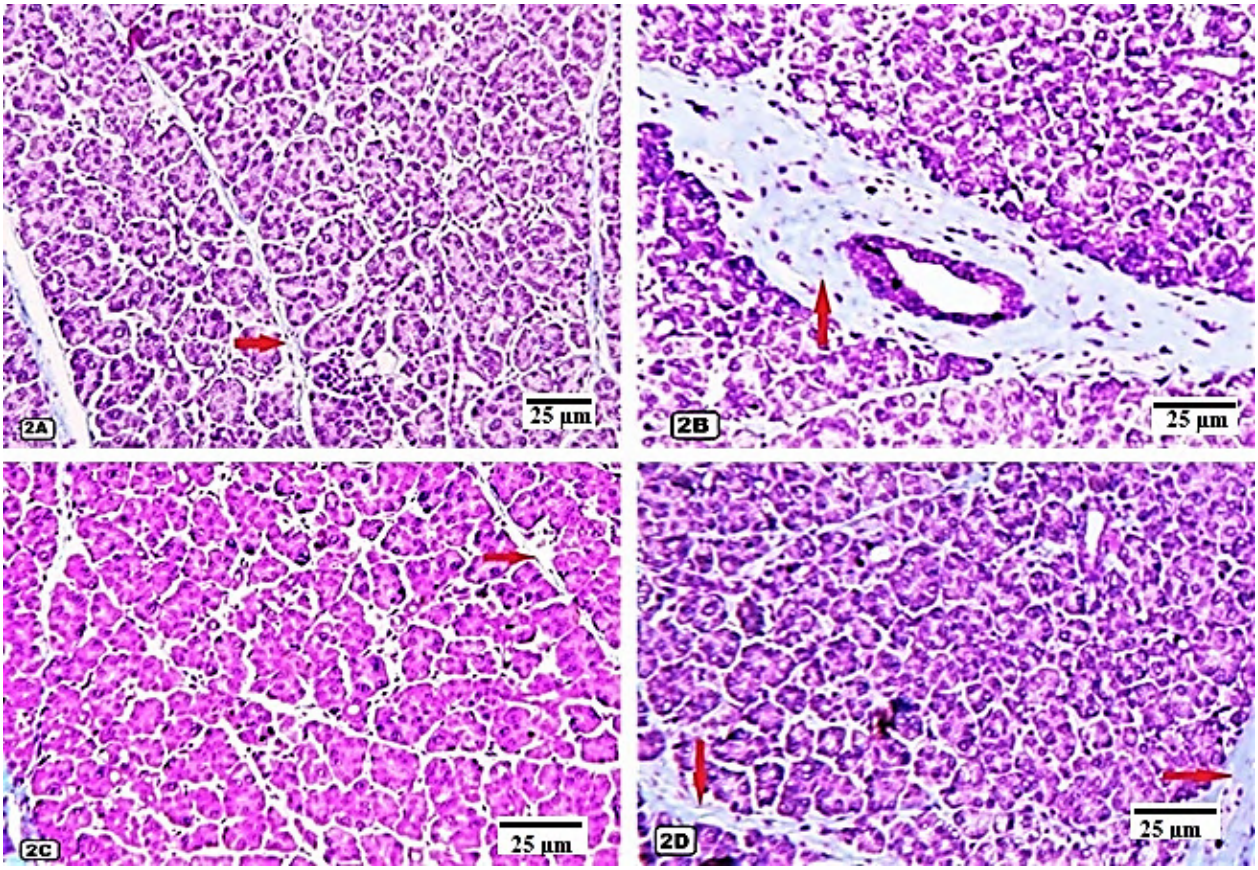

Fig. 2.- Masson's trichrome stain for all groups. ×200. Scale bars = 25 µm. **(2A)**: Section of the parotid gland of a sham control rat showing few blue stained collagen fibers (red arrow) in the connective tissue septa between the lobules. **(2B)**: Section from Group II showing extensive blue collagen surrounding lobules, interlobular ducts, and blood vessels. (red arrow). **(2C)**: Section from Group III showing few blue collagen fibres in inter and intra lobular septa (red arrow). **(2D)**: Section from Group IV showing moderate amount of blue collagen fibres in inter and intra lobular septa (red arrows).

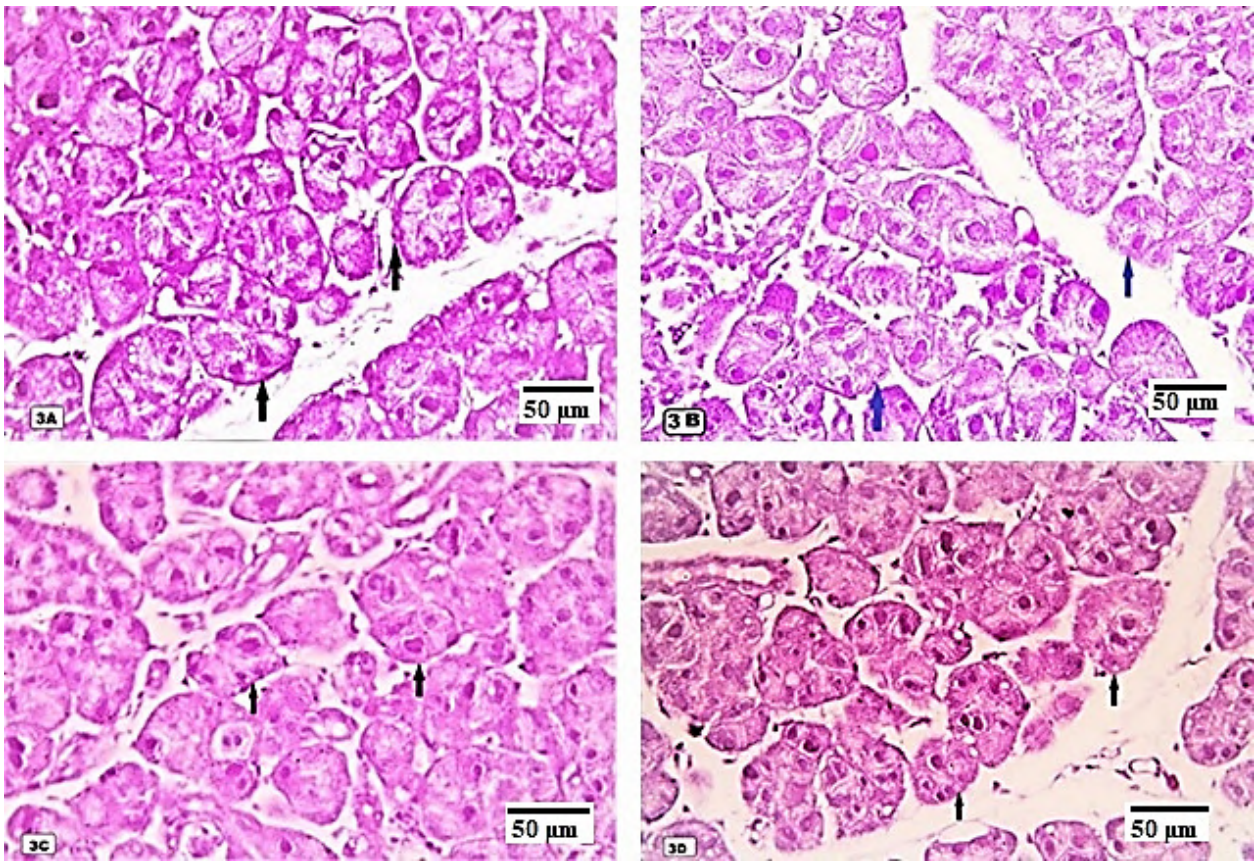

Fig. 3.- PAS reaction of all groups. ×400. Scale bars = 50 µm. **(3A)**: Section of the parotid gland of a control rat showing obvious positive PAS reaction in the basement membrane of the acini (black arrows). **(3B)**: Section of the parotid gland of irradiated group showing mild PAS reaction in the basement membrane of acini (blue arrows). **(3C)**: Section of the parotid gland of a rat in irradiated and with vit. C showing strong positive PAS reaction in basement membrane of almost acini (black arrows). **(3D)**: Section of the parotid gland of a rat in operated and withdrawal group showing moderate PAS reaction in basement membrane of almost acini (black arrows).

the acini and intra-lobular ducts more or less as control (Fig. 4C).

G*roup IV (irradiated +Withdrew group)*: Sections showed α-SMA immunoreaction in multiple MECs at the periphery of the acini and intra-lobular ducts more or less as control and less than Group II (Fig. 4D).

(Ki-67)

Group I (sham control group): Immunoreaction to Ki-67 showed positive immune reaction in very few nuclei (nucleoplasm) of acinar cells and the cells lining an intra-lobular duct (Fig. 5A).

G*roup II (irradiated group)*: Immunohistochemical examination showed of Ki- 67 slightly increased in positive reaction nuclei of the lining cells of acini and some cells lining an interlobular duct (Fig. 5B).

G*roup III (irradiated + Vit C group)*: Immunoreaction to Ki-67 in few nuclei of acinar cells nearly as control group (Fig. 5C).

G*roup IV (irradiated + Withdrew group)*: Immunoreaction to Ki-67 in few nuclei of acinar cells more or less as in Group II (Fig. 5D).

Morphometric analysis

The statistical analysis between the studied groups revealed that there was a significant increase in the mean number of vesicular nuclei ($P < 0.05$) in the sham control group (I) when compared with exposure group (II). But no significant differences ($P > 0.05$) were found when group III and group IV were compared, or when the last two groups compared with sham control group (Table 1). No significant increase ($P > 0.05$) in the mean area % of collagen fibers stained by Masson's trichrome were found when compared between sham control group and group III or group IV. But a significant increase ($P < 0.05$) in the mean area % of collagen fibers stained by Masson's trichrome of exposure group II was found when compared with shame control group (Table 1). No significant difference was found ($P > 0.05$)

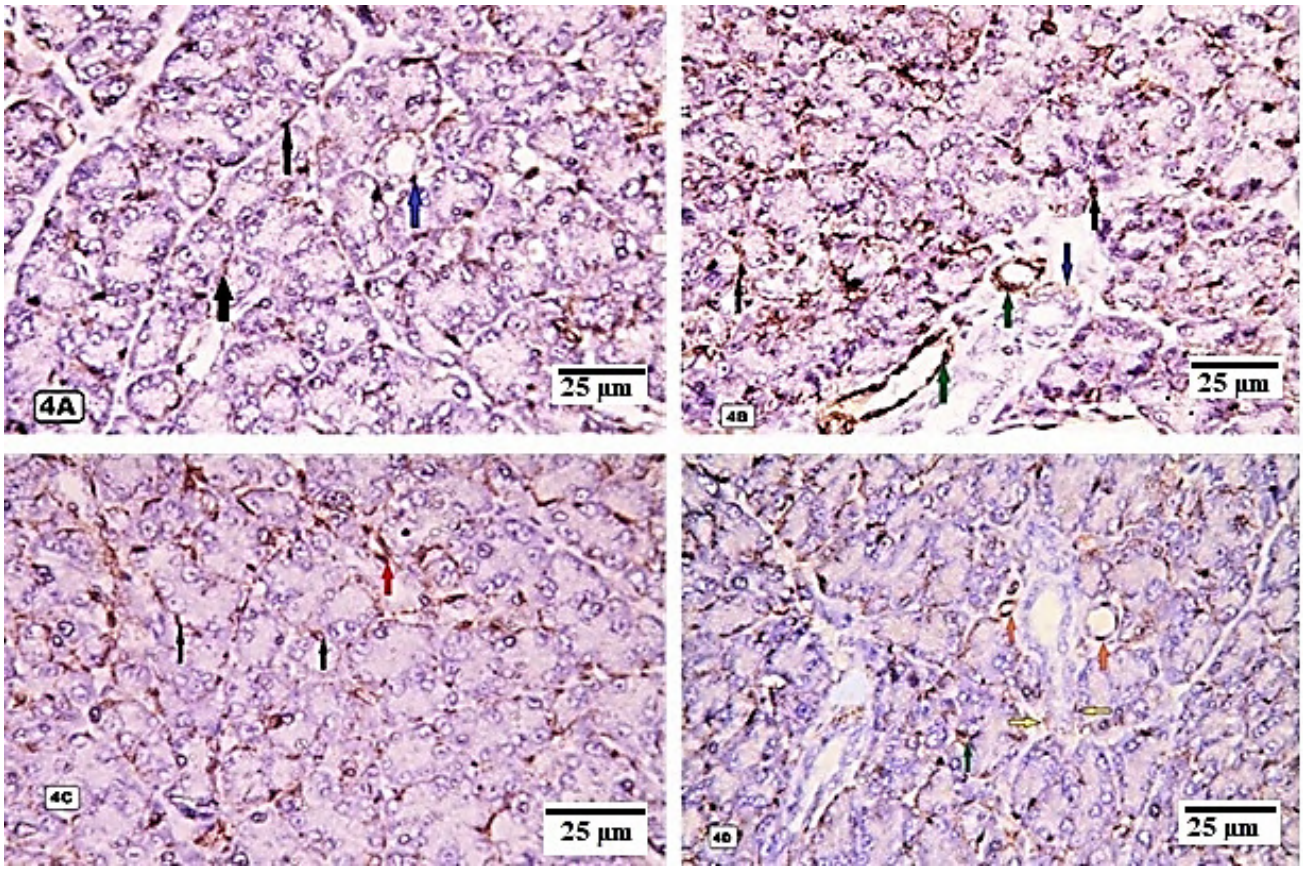

Fig. 4.- α-SMS immunoreaction of all groups. ×200. Scale bars = 25 µm. **(4A)**: Section of the parotid gland of a control rat showing a moderate α-SMA immunoreaction in multiple MECs at the periphery of the acini (black arrows) and intralobular ducts (blue arrow). **(4B)**: Section of the parotid gland of a rat in irradiated group showing an apparent increase in immune reaction for α-SMA in MECs at the periphery of the acini (black arrows), interlobular ducts (blue arrow) and in the lining of two blood vessels (green arrow) is also seen. **(4C)**: Section of the parotid gland of a rat in irradiated and with vit. C group showing a moderate α smooth muscle actin immunoreaction at the periphery of the acini (black arrows) intralobular ducts (red arrow), nearly as a control group. **(4D)**: Section of the parotid gland of a rat in irradiated and then withdrawal group showing α-SMA immunoreaction in multiple MECs at the periphery of the acini (green arrows) and intralobular ducts (yellow arrow) and in the lining of blood vessels (red arrows) less more than control group but less than irradiated group.

in the mean area % of PAS reaction in group III compared with sham control Groups I, and IV. But a significant decrease (P <0.05) in the mean area % of PAS reaction in Exposure group II was found when compared with sham control group I (Table 1). A significant increase (P <0.05) in the mean number of positive α-SMA in cytoplasm of MECs and mean number of positive Ki- 67 nuclei expression in exposure group II was found compared with sham control group. But no significant differences (P >0.05) were found when compared between group III and group IV or when the last two groups were compared with sham control group (Table 1).

Table 1. Mean count of vesicular nuclei, mean area percentage of Masson trichrome stained collagen fibers and PAS +ve staining, area percentage of α-SMA +ve MECs and Ki67 + in all studied groups.

	Group I (sham control)	**Group II (irradiated)**	**Group III (irradiated+ vit C)**	**Group IV (irradiated + withdrew)**
Count of vesicular nuclei	11.5 ± 1.3	6.1 ± 0.5*	10.7 ± 1.4 #	9.6± 1.2#
Collagen fibers	2.9 ± 0.5	7.8± 1.1*	3.4± 0.3 #	3.9±0.6 #
PAS +ve staining	4.1± 0.3	2. 6 ± 0.4*	3.8 ± 0.6#	3.2 ± 0.5#
SMA+ve MECs	2.47± 0.2	4.96 ± 0.7*	2.27± 0.3 #	2.52±0.2 #
Ki-67 +ve nuclei	0.35 ±0.04	0.95±0.20*	0.31 ±0.04#	0.38±0.05#

*significant <0.05

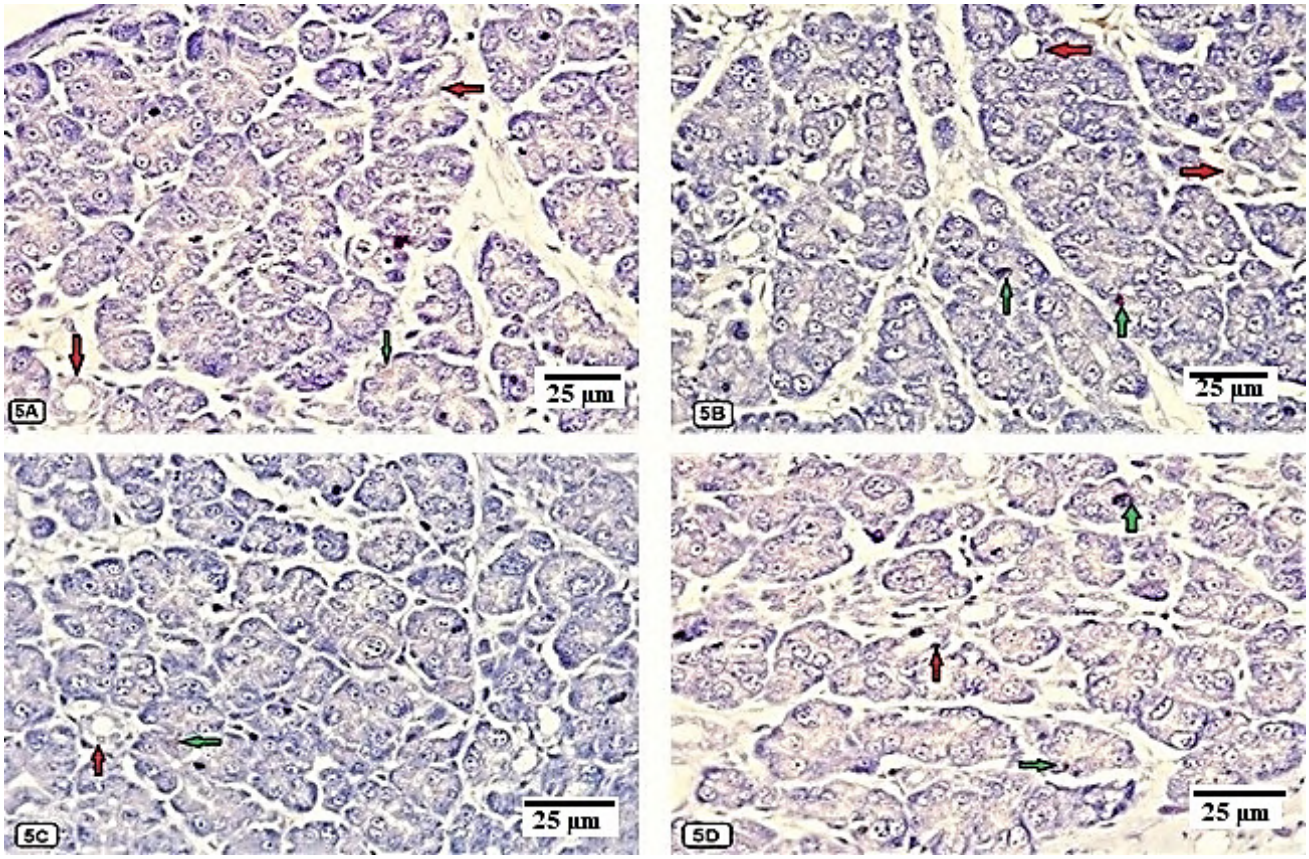

Fig. 5.- Ki-67 immunoreaction of all groups. x 200. Scale bars = 25 µm. **(5A)**: Section of the parotid gland of a rat of control group showing weak +ve Ki-67 immunoreaction in few nuclei of acinar cells (green arrow) but -ve reaction in the cells lining in an interlobar duct (red arrow). **(5B)**: Section of the parotid gland of group II (irradiated) rat showing moderate +ve Ki-67 immunoreactivity in nuclei of some cells of acini (green arrows) and also +ve in some nuclei of duct cells (red arrows). **(5C)**: Section of the parotid gland of a rat in Group III showing nearly to the control group in a few weak +ve Ki-67 immunoreaction in few nuclei of acinar cells (green arrow) but -ve reaction in the cells lining in an interlobar duct (red arrow). **(5D)**: Section of the parotid gland of rats in Group IV showing Ki- 67 immunoreaction in few nuclei (green arrow) of acinar cells.

Biomolecular analysis

Salivary amylase and total protein

There was a significant reduction in the secretory activity of the parotid gland (P <0.05) regarding the values of total protein and amylase activity in exposure group II compared with sham control groups I. But both Vit C administration and radiation withdrawal significantly (p<0.05) retain the secretory activity. No significant differences (P >0.05) between group I, III and group IV were found (Table 2).

Parotid tissue MDA and SOD

Exposure to mobile phone radiation significantly (p<0.05) increase the oxidative stress within the parotid tissue denoted by increase MDA levels and decrease SOD levels while Vit C administration

Table 2. Mean value of total protein (µg/ml), mean value of amylase activity (ng /ml), mean value of MDA (moL/mg tissue) and SOD (U/ml) in all studied groups.

	Group I (sham control)	Group II (irradiated)	Group III (irradiated + vit C)	Group IV (irradiated+ withdrew)
Total protein	83.7±3.8	60.9±3.6 *	78.6±9.8#	71.7±9.8#
Amylase activity	220.1±9.9	165.9±7.2 *	209.2±9.1#	198.7±8.8#
MDA	4.8± 0.3	9.9± 1.2 *	5.2±0.5#	5.9±0.6#
SOD	29.8± 5.57	12.65± 1.45 *	22.5±5.07#	23.3±3.22#

*significant P <0.05 compared to control
#significant P <0.05 compared to irradiated group

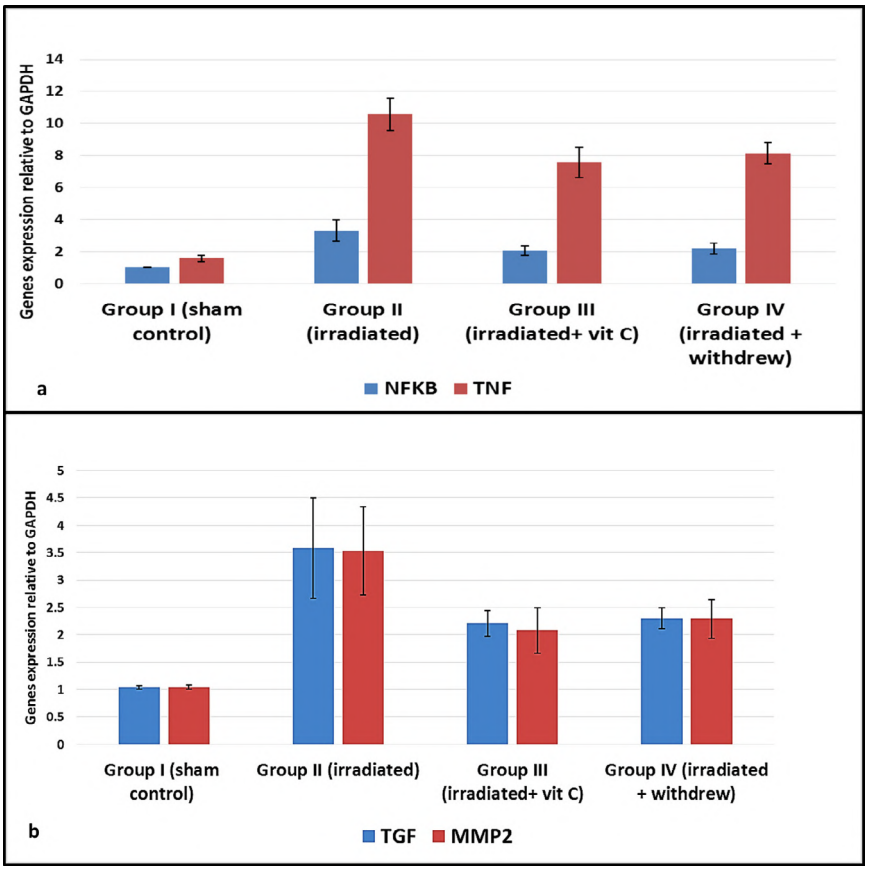

Fig. 6.- a: Inflammatory cytokines NFKB, TNF, **b:** fibrotic genes TGF, MMP2 expressions with the highest expression in irradiated group and significant reduction in their expression with Vit. C administration also after withdrawal from radiation exposure.

and radiation withdrawal significantly ($p<0.05$) restore the antioxidant/oxidant capacity through increase SOD and decrease MDA. No significant differences ($P >0.05$) between group, III and group IV (Table 2) were found.

PCR results

Mobile phone radiation exposure significantly ($p<0.05$) up-regulates the expression of inflammatory cytokines NF KB, TNF and the fibrotic markers TGF, MMP2 within the parotid tissue while Vit C administration and radiation withdrawal significantly ($p<0.05$) decrease their expression. No significant differences ($P >0.05$) between group, III and group IV were found (Fig. 6).

DISCUSSION

In the past two decades, a continuous accumulation of knowledge about the possible health risks from exposure to the electromagnetic fields radiated from phones has been observed (Helal and Abdelrahman, 2012), raising concerns about their long-term effect on the parotid salivary gland (Ghadhban and Mhaibes, 2018).

The parotid gland is the largest salivary gland and, together with the submandibular salivary gland, produces the majority of saliva volume. The anatomical site of the parotid gland makes it more sensitive to EMR than the submandibular gland (Zhang et al., 2020a). The parotid gland is composed mainly of serous cells. Thus, injury to the parotid gland is the main cause of xerostomia (Altayeb, 2018). This is the main reason for this study's choice of the parotid gland. In this study, the parotid glands of rats that were exposed to 2100 MHz radiofrequency radiation from a generator that looked like a 3G cell phone changed a lot histologically compared to rats that were put in a sham control group. The changes were observed in the connective tissue, epithelial cells, and interstitial spaces. Mononuclear cell infiltration, dilated, congested blood vessels, dark stained nuclei, and vacuoles in the cytoplasm of acinar cells were observed, and this coincides with previous studies reporting that exposure to EMR from a mobile phone of the third generation, with a frequency of

2100 MHz, results in changes in the histological structure of parotid gland in rats (Maher et al., 2020). Other research confirmed that EMR had non-thermal effects that led to more oxidative stress in the tissue that was exposed to it (Imam et al., 2021). Another study suggested that exposure to such EMR leads to an increase in the level of free radicals (Carvalho Siqueira et al., 2016). According to additional studies (Aydogan et al., 2015), oxidative stress and several pathophysiological changes after irradiation cause damage to the parotid glands. ROS play a crucial role in signal transduction, regulating cellular functions like cell death and growth (Kivrak et al., 2017). Excessive ROS production attenuates the cells' antioxidant capacity, creating a state of oxidative stress and leading to severe cellular damage (Dasdag et al., 2016). A previous study reported that ROS levels sharply increased in salivary glands after EMR (Aydogan et al., 2015).

In the present study, the connective tissue stroma of the parotid glands of radiation showed dense interlobular and moderate intralobular fibrosis, characterized by a significant increase in the mean area percentage of collagen fibers. These findings were in accordance with the authors (Maher et al., 2020), who reported that fibrosis is common in advanced EMR injuries. Radiation can cause oxidative stress, which can strengthen the inflammatory response and cause more inflammatory cytokines to be made (Siqueira et al., 2016). The current study showed cellular infiltration and congested blood vessels in some areas in parotid sections that could be part of the inflammatory response. MDA is a byproduct of polyunsaturated fatty acid oxidation, and is a marker of oxidative stress-mediated lipid peroxidation (Gaweł et al., 2004). In the current study, there is a significant increase in tissue MDA and a decrease in SOD levels in the radiation-exposed group compared to a control group, with a significant reduction in its level after Vit C supplementation. It has been reported that vitamins E and C have antiapoptotic activity through the reduction of MDA and restoration of SOD in many organs. (Younus, 2018). Previous studies suggested that SOD partially protects against injuries produced by head-and-neck irradiation (Nagler et al., 2000). Cell phone radiation exposure increases the concentration of peroxynitrite, and superoxide that destroy DNA, proteins, and lipids and consequently result in apoptosis and necrosis (Aydogan et al., 2015). Our study showed a significant decrease in the mean area per cent of PAS-positive material in irradiated rats compared to the control group due to the depletion of basement membrane at some injured areas in response to the toxic effects of EMR, as explained before. This was preserved as nearly in groups III and IV as in the control group. The latter was the role of vitamin C in cell protection from breakthrough inflammation. These findings agreed with those of other studies (Anan et al., 2021).

Saliva is an underused diagnostic tool, but in the last three decades it has gained much attention, as it is easy and non-invasive in its collection (Kaufmann and Lamster, 2002). We found a significant decrease in total protein and amylase activity in the saliva of the exposed group compared to the control group, while there was a significant increase in their levels in the Vit-C-supplemented group. Goldwein and Aframian (2010) reported that microwave radiation exposure decreases salivary protein secretion as a result of continuous and cumulative glandular damage. More studies reported that higher salivary levels of amylase, LDH and MDA were detected in high mobile users than low mobile users. (Shivashankara et al., 2015).

In recent years, plant-derived antioxidants have received increased attention due to their potent antitoxic effect, low toxicity, and lower cost (Liu et al., 2021). Previous studies reported that patients' saliva with prolonged phone exposure has decreased flow rate, pH, buffer capacity, and total protein (Hashemipour et al., 2014; Madhukar et al., 2019). In this study, the MECs demonstrated by immunohistochemistry for αSMA revealed a highly significant increase in α-SMA immunoreactivity around acini and intra-lobular ducts in the irradiated group. The MECs undergo proliferative and morphological changes during the atrophy and regeneration of acinar cells, with subsequent increases in size and number. In stressful conditions, it seems that gland function recovery requires increasing the surviving cells' secretory

capacity. More MECs need to squeeze the accumulated secretion (Altayeb, 2018).

In the present study, an increase in Ki-67 immuno-reaction in the parotids of rats Exposed to EMR was in accordance with a previous study that reported that exposure to EMF radiation from many sources, like cell phones, has a proliferative effect on salivary acini, and revealed that the increase in Ki-67 expression was directly related to the severity of the EMF exposure. Also, several previous studied reported the relation between benign and malignant tumor and long-term exposure of phone radiation (Hantke et al., 2002).

In this study, Ki-67 immunoreaction appeared nearly as a control in GIII, in which rats took Vit C. Previous studies explained that Vit C could restore normal cell proliferation of the salivary gland (Maher et al., 2020). Several studies over the past decade have reported that increasing usage of mobile phones could have possible carcinogenic effects as a result of exposure to radiofrequency (Choi et al., 2020).

Vitamin C is a powerful antioxidant that helps to reduce oxidative stress molecules in the body, such as MDA; vitamin C acts as a redox buffer that could reduce and neutralize reactive oxygen species. Various studies explained that electromagnetic waves emitted from mobile phones (900MHz) affect cell proliferation and apoptosis through the induction of ROS. ROS are scavenged by SOD, glutathione peroxidase, and catalase (Ashor et al., 2014; Ahmad et al., 2016).

Ki-67 is a human nuclear protein important for cell proliferation (Scholzen and Gerdes, 2000). The results of the current study indicate an increase in immune expression of Ki67 protein in glandular tissue cells. This indicates an increased ability of these cells for mitotic division in response to oxidative stress damage.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that influences salivary gland homeostasis and development. TGF-β induces the ECM deposition by inducing the biosynthesis of fibronectin and collagens (Leask and Abraham, 2004) in addition to protease inhibitors. Moreover, TGF-β1 enhances epithelial-mesenchymal transition with increased formation of myofibroblasts. Salivary gland tissue damage from inflammation or radiation exposure could result in reparative TGFβ-induced ECM production (Zavadil and Böttinger., 2005). In the current study, there is significant upregulation in TGF gene expression in parotid tissue of the exposed group compared to a control group, with a significant reduction in its expression after Vit C supplementation. This is in agreement with a previous study, which reported that irradiation of the submandibular salivary gland causes a significant decrease in salivary secretion and amylase activity, but the elevation of MDA with an increase in collagen deposition and TGF expression (Xu et al., 2016). Further studies reported that treating diabetic rats with vitamin C prevents the increase in TGF in glomerular and cortical tissues (Craven et al., 1997).

TGF-β-signaling has a crucial role in fibrosis, including salivary gland fibrosis. Previous studies mentioned that TGF-β/BMP-signaling molecules could be a promising anti-fibrotic drug target (Zhang et al., 2020b). A previous study reported 10-fold increase in salivary TGF-β protein expression in patients with radiation-induced salivary dysfunction (Hakim et al., 2011, Spiegelberg et al., 2014). Another study revealed that TGF-β1 expression increased after irradiation in an experimental mouse model and then decreased after hyperbaric oxygen therapy (Hoesel and Schmid, 2013).

NF-κB is a central factor in cell differentiation, proliferation, inflammation, stress response, and cell death. NF-κB is activated by many stimuli and a network of signaling pathways, which influence each other (Freudlsperger et al., 2013). TGF-β activates NF-κB signaling involving TAK1 and IKK kinases leading to IκBα phosphorylation, nuclear translocation of NF-κB and activation of NF-κB downstream targets (Kim et al., 2016). In the parotid tissue of the exposed group, NF-κB and TNF gene expression were significantly higher than in the control group. However, these genes were significantly lower in expression after vitamin-C supplementation. Our results are in accordance with Matsuno et al. (2002), who reported a significant increase in the expression of inflammatory cytokines NF-κB, IκB-α, and fibrotic marker TGF-β1 in salivary gland exposed to radiation in experimental rats.

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that is found to be increased in numerous autoimmune or inflammatory diseases (Sullivan et al., 2005). A previous study reported that TNF-α up-regulates TGF-β1 expression in primary mouse lung fibroblasts (Han et al., 2001). It has been reported that TNF-alpha stimulates activation of proMMP2 in human skin through NF-kB (Brew and Nagase, 2010). Matrix metalloproteinases (MMPs) are a group of enzymes that play an important role in the turnover of ECM in normal and pathological processes. Herein we found significant up-regulation of MMP2 in the exposed group, which retains to nearly normal in Vit C supplemented group, which is in accordance with Brew and Nagase (2010), who reported that Vit C and E were shown to reduce MMP expression and activity in human dermal fibroblasts after UVA irradiation.

CONCLUSION

Radiofrequency emitted from mobile phones has harmful effects on different organs and tissues. Exposure to these waves affects the secretory function of the parotid gland due to oxidative stress and the release of inflammatory cytokines with subsequent fibrosis. In addition to accelerated cellular proliferation to replace the damaged tissues, the currently used drugs have many intolerable side effects, so we urgently need to discover a safe method of protection against phone exposure side effects. Vitamin C is a powerful antioxidant that helps in prophylactic protection against these harmful effects by ameliorating oxidative stress.

Recommendation: The study recommended the need for more work on changing the behaviors that constitute a danger to users of mobile phones, and more research must be done to adopt preventive measures, such as the reduction and rationalization of direct exposure to mobile use. Long-term experimental and clinical studies are needed to clarify the effect of radiofrequency radiation emitted from 3G mobile phones on parotid tissue.

REFERENCES

AHMAD AG, KOBRA S, DARYOUSH MN (2016) Protective effects of vitamin E consumption against 3MT electromagnetic field effects on oxidative parameters in substantia nigra in rats. *Basic Clin Neurosci*, 7(4): 315-322.

ALTAYEB ZM (2018) Possible protective role of green tea extract on male rat parotid gland in high fat diet induced obesity (histological study). *J Med Histol*, 2(1): 69-80.

ANAN H, GAWISH M, AMER M, IBRAHIM N (2021) Effects of low magnetic irradiation on morphology and ultrastructure of parotid glands in rats and amelioration by vitamin E. *J Cytol Histol*, 3: 139-142.

ASHOR AW, LARA J, MATHERS JC, SIERVO M (2014) Effect of vitamin C on endothelial function in health and disease: a systematic review and meta-analysis of randomised controlled trials. *Atherosclerosis*, 235(1): 9-20.

AYDOGAN F, UNLU I, AYDIN E, YUMUSAK N, DEVRIM E, SAMIM EE, OZGUR E, UNSAL V, TOMRUK A, OZTURK GG, SEYHAN N (2015) The effect of 2100 MHz radiofrequency radiation of a 3G mobile phone on the parotid gland of rats. *Am J Otolaryngol*, 36: 39-46.

BREW K, NAGASE H (2010) The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. *Biochim Biophys Acta*, 1803(1): 55-71.

CARVALHO SIQUEIRA E, ALVIM DE SOUZA FT, FERREIRA E, PEDRA SOUZA R, COSTA MACEDO S, FRIEDMAN E, VINICIUS GOMEZ M, CAVALIÉRI GOMES C, SANTIAGO GOMEZ R (2016) Cell phone use is associated with an inflammatory cytokine profile of parotid gland saliva. *J Oral Pathol Med*, 45: 682-686.

CHOI YJ, MOSKOWITZ JM, MYUNG SK, LEE YR, HONG YC (2020) Cellular phone use and risk of tumors: systematic review and meta-analysis. *Int J Environ Res Public Health*, 17(21): 8079.

CRAVEN PA, DERUBERTIS FR, KAGAN VE, MELHEM M, STUDER RK (1997) Effects of supplementation with vitamin C or E on albuminuria, glomerular TGF-beta, and glomerular size in diabetes. *J Am Soc Nephrol*, 8(9): 1405-1414.

DASDAG S, AKDAG MZ (2016) The link between radiofrequencies emitted from wireless technologies and oxidative stress. *J Chem Neuroanat*, 75: 85-93.

EL-AKABAWY G, EL-KHOLY W (2014) Neuroprotective effect of ginger in the brain of streprozotocin-induced diabetic rats. *Ann Anat*, 169: 119-128.

EL-BEDIWI A, ATTALL F, SAAD EM, EID F (2011) Effects of electromagnetic radiation produced by mobile phone on some visceral organs of rat. *J Med Sci*, 11: 256-260.

EMSLEY R, DUNN G, WHITE I (2010) Mediation and moderation of treatment effects in randomized controlled trials of complex interventions. *Stat Methods Med Res*, 19(3): 237-270.

FREUDLSPERGER C, BIAN Y, CONTAG WISE S, BURNETT J, COUPAR J, YANG X, CHEN Z, VAN WAES C (2013) TGF-β and NF-κB signal pathway cross-talk is mediated through TAK1 and SMAD7 in a subset of head and neck cancers. Oncogene, 32(12): 1549-1559.

GHADHBAN R, MHAIBES A (2018) Study effect electromagnetic field and mobile phone radiation on some hematological, biochemical and hormonal parameters in female rats. *Basrah J Vet Res*, 17: 155-164.

GOLDWEIN O, AFRAMIAN DJ (2010) The influence of handheld mobile phones on human parotid gland secretion. *Oral Dis*, 16(2): 146-150.

HAKIM SG, RIBBAT J, BERNDT A, RICHTER P, KOSMEHL H, BENEDEK GA, JACOBSEN HC, TRENKLE T, SIEG P, RADES D (2011) Expression of Wnt-1, TGF-β and related cell-cell adhesion components following radiotherapy in salivary glands of patients with manifested radiogenic xerostomia. *Radiother Oncol*, 101(1): 93-99.

HAN YP, TUAN TL, WU H, HUGHES M, GARNER WL (2001) TNF-alpha stimulates activation of pro-MMP2 in human skin through NF-(kappa)B mediated induction of MT1-MMP. *J Cell Sci*, 114(Pt 1): 131-139.

HANTKE B, LAHMANN C, VENZKE K, FISCHER T, KOCOUREK A, WINDSOR LJ, BERGEMANN J, STÄB F, TSCHESCHE H (2002) Influence of flavonoids and vitamins on the MMP- and TIMP-expression of human dermal fibroblasts after UVA irradiation. *Photochem Photobiol Sci*, 1(10): 826-833.

HASHEMIPOUR M, YARBAKHT M, GHOLAMHOSSEINIAN A, FAMORI H (2014) Effect of mobile phone use on salivary concentrations of protein, amylase, lipase, immunoglobulin A, lysozyme, lactoferrin, peroxidase and C-reactive protein of the parotid gland. *J Laryngol Otol*, 128(5): 454-462.

HELAL M, ABDELRAHMAN M (2012) Ki-67 expression on major salivary gland exposed to mobile cell phone radiation. *EDJ*, 58(3).

HOESEL B, SCHMID JA (2013) The complexity of NF-κB signaling in inflammation and cancer. *Mol Cancer,* 12: 86.

IMAM HASAN, TANJINA AMIN, RAFIQUL ALAM, MOHAMMAD RAFIQUL ISLAM (2021) Hematobiochemical and histopathological alterations of kidney and testis due to exposure of 4G cell phone radiation in mice. *Saudi J Biol Sci*, 28(5): 2933-2942.

JELODAR G, NAZIFI S, AKBARI A (2013) The prophylactic effect of vitamin C on induced oxidative stress in rat testis following exposure to radio frequency wave generated by a BTS antenna model. *Electromagn Biol Med*, 32(3): 409-416.

JOHANSSON I, LINDER J, BRATT P (1989) Comparison of saliva secretion rate and composition in the rat using a pentobarbital or a neuroleptanalgesic type of anaesthesia. *Caries Res*, 3: 75-77.

KAUFMANN E, LAMSTER IB (2002) The diagnostic applications of saliva, a review. *Crit Rev Oral Biol Med*, 13: 197-212.

KIM JH, KIM KM, JUNG MH, JUNG JH, KANG KM, JEONG BK, KIM JP, PARK JJ, WOO SH (2016) Hoon protective effects of alpha lipoic acid on radiation-induced salivary gland injury in rats. *Oncotarget*, 7(20): 29143-29153.

KIVRAK EG, YURT KK, KAPLAN AA, ALKAN I, ALTUN G (2017) Effects of electromagnetic fields exposure on the antioxidant defense system. *J Microsc Ultrastruct*, 5: 167-176.

LEASK A, ABRAHAM DJ (2004) TGF-beta signaling and the fibrotic response. *FASEB J,* 18: 816-827.

LIU Z, DONG L, ZHENG Z, LIU S, GONG S, MENG L, XIN Y, JIANG X (2021) Mechanism, prevention, and treatment of radiation-induced salivary gland injury related to oxidative stress. *Antioxidants (Basel)*, 10(11): 1666.

LUO. S, TAN. W, DENG W, ZHUANG S, LUO J (2005) Expression of albumin, IGF1, IGFBP-3 in tumor tissues and adjacent non-tumor tissues of hepatocellular carcinoma patients with cirrhosis. *World J Gastroenterol*, 11: 4272-4276.

MADHUKAR VASHISTHA, RITESH KATIYAR, PRIYADERSHINI RANGARI (2019) The influence of cell phone radiation on saliva: an observational study. *Int J Med Sci Diagn Res*, 3(7): 22-27.

MAHER AL-ASSAF, CHARIF BARAKAT, MAJID G.A. ABO FAKHER, ADNAN ALMALKI, MOURAD A. ABO FAKHER (2020) Effects of mobile phone radiation on parotid gland: immunohistochemical study. *J Stoma*, 73(3): 112-117.

MATSUNO H, YUDOH K, KATAYAMA R, NAKAZAWA F, UZUKI M, SAWAI T, YONEZAWA T, SAEKI Y, PANAYI GS, PITZALIS C, KIMURA T (2002) The role of TNF-alpha in the pathogenesis of inflammation and joint destruction in rheumatoid arthritis (RA): a study using a human RA/SCID mouse chimera. *Rheumatology (Oxford)*, 41(3): 329-337.

NAGLER RM, REZNICK AZ, SLAVIN S, NAGLER A (2000) Partial protection of rat parotid glands from irradiation-induced hyposalivation by manganese superoxide dismutase. *Arch Oral Biol*, 45(9): 741-747.

ROSADO MM, NASTA F, PRISCO MG, LOVISOLO GA, MARINO C, PIOLI C (2014) Effects of GSM▁modulated 900 MHz radiofrequency electromagnetic fields on the hematopoietic potential of mouse bone marrow cells. *Bioelectromagnetics*, 35: 559-567.

SCHOLZEN T, GERDES J (2000) The Ki-67 protein: from the known and the unknown. *J Cell Physiol*, 182(3): 311-322.

SHIVASHANKARA AR, JOY J, SUNITHA V, RAI MP, RAO S, NAMBRANATHAYIL S, BALIGA MS (2015) Effect of cell phone use on salivary total protein, enzymes and oxidative stress markers in young adults: a pilot study. *J Clin Diagn Res*, 9(2): BC19-22.

SIQUEIRA EC, DE SOUZA FTA, FERREIRA E, SOUZA RP, COSTA MACEDO S, FRIEDMAN E, GOMEZ MV, CAVALIERI GOMES V, GOMEZ RS (2016) Cell phone use is associated with an inflammatory cytokine profile of parotid gland saliva. *J Oral Pathol Med*, 45: 682-686.

SPIEGELBERG L, SWAGEMAKERS SMA, VAN IJCKEN WFJ, OOLE E, WOLVIUS EB, ESSERS J, BRAKS JAM (2014) Gene expression analysis reveals inhibition of radiation-induced TGF beta-signaling by hyperbaric oxygen therapy in mouse salivary glands. *Mol Med,* 20: 257-269.

SULLIVAN DE, FERRIS M, POCIASK D, BRODY AR (2005) Tumor necrosis factor-alpha induces transforming growth factor-beta1 expression in lung fibroblasts through the extracellular signal-regulated kinase pathway. *Am J Respir Cell Mol Biol*, 32(4): 342-349.

SUVARNA SK, LAYTON C, BANCROFT JD (Eds.) (2013) Bancroft's *Theory and Practice of Histological Techniques*. 7th ed., ch10. Churchill Livingstone, Philadelphia, pp 172-186, 382-434, 493, and 239-270.

XU L, YANG X, CHEN J, GE X, QIN Q, ZHU H, ZHANG C, SUN X (2016) Simvastatin attenuates radiation-induced salivary gland dysfunction in mice. *Drug Des Devel Ther,* 10: 2271-2278.

YOUNUS H (2018) Therapeutic potentials of superoxide dismutase. *Int J Health Sci*, 12(3): 88-93.

ZAVADIL J, BÖTTINGER EP (2005) TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene,* 24: 5764-5774.

ZHANG T, LIU C, MA S, GAO Y, WANG R (2020a) Protective effect and mechanism of action of rosmarinic acid on radiation-induced parotid gland injury in rats. *Dose Response*, 18(1): 1559325820907782.

ZHANG X, YUN JS, HAN D, YOOK JI, KIM HS, CHO ES (2020b) TGF-β pathway in salivary gland fibrosis. *Int J Mol Sci*, 21(23): 9138.