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ORIGINAL ARTICLE

Clove bud oil effect on reactive oxygen species released due to mobile phones electromagnetic radiations on the parotid gland of albino rats

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Abstract

Background: Mobile phones have become widespread around the globe. These devices emit electromagnetic radiations (EMR) which induce oxidative stresses and reactive oxygen species (ROS) in the body tissues. Nowadays, natural antioxidants are widely used to counteract the ROS including the clove bud essential oil.

Aim: The aim of the current study is to investigate the effect of clove bud oil in protecting the parotid glands against the harmful effect of mobile phones EMR.

Methodology: Twelve adult male albino rats were randomly distributed into two groups, six as positive control group (A) exposed to the mobile phone EMR and six as experimental group (B) exposed to EMR followed by administration of clove bud oil as a treatment for 30 successive days. The parotid glands were dissected for histological examination, immunohistochemical localization of myeloperoxidase (MPO), biochemical examination for the measurement of nuclear factor kappa beta (NF-κB), and histomorphometric analysis of diameter and number of serous acini and area percentage of MPO.

Results: The histological examination of the parotid gland of Group (A) revealed loss of the normal architecture of the gland, while Group (B), the architecture was closer to the normal. Statistically, significant decrease in the mean values of MPO and NF-κB in Group (B) as compared to Group (A). Histomorphometric analysis revealed significant decrease in the diameter and number of serous acini in Group (A) as compared to Group (B).

Conclusions: Clove bud oil had a positive role against the ROS produced by the mobile phones EMR as it did a noticeable improvement in the structure of the parotid gland due to its antioxidant activity.

Clinical Significance: The clove bud essential oil could be introduced as a natural food preservative and as food additive due to its powerful antioxidant capacity.

Introduction

Over the past few decades, the development of wireless technology has rapidly flourished worldwide, including the increase in the use of wireless telephone communication, which raised the awareness about its health risks. The number of mobile users all over the world increased above 6,800,000,000 and the number is rising at a very fast rate. Nowadays, all the age groups use the mobile phones and the average use for the mobile phone is 90 min/day for each person.[1] According to the statistics released by the Ministry of Communications and Information Technology in Egypt, the mobile phone users reached 94.59 million in September 2018.[2]

The mobile phones emit electromagnetic radiations (EMR) which have hazardous effects on the human health in general and on the parotid gland specifically,[3] due to its proximity to the mobile phone during its use.[4] These radiations affect the biological systems by induction of oxidative stresses in the tissues,

thus producing reactive oxygen species (ROS) and increasing the free radicals which cause lipid peroxidation (LPO) and changes the antioxidant defense systems of human tissues.[5] Radiofrequency EMR is classified according to the World Health Organization/ International Agency for Research on Cancer as possibly carcinogenic to humans and associates it with wireless phone use.[6]

The herbal medicines have been popularly used for prevention and treatment of diseases, and in recent years, the herbal remedies are used as primary health care with minimum side effects around the world.[7] The clove essential oil is isolated from the buds of Eugenia caryophyllata. It is widely used for its medical properties in dental care. Being an anti-inflammatory,[8] antiseptic, and analgesic, the undiluted form is rubbed on the gingiva to treat toothache and as a powerful antioxidant. The antioxidant activity of clove bud oil is mainly attributed to the eugenol where its mechanism of action may occur through scavenging the radicals and chelating metal ions.[9]

From the forementioned knowledge, it is obvious that the mobile phone EMR may have deleterious effects over various body organs in general and over the parotid gland in specific although the data about the health risks are limited, the absence of evidence of harm should not necessarily be interpreted as evidence that no harm exists. Therefore, the current study will focus on investigating the production of ROS in the parotid gland of albino rats after their exposure to mobile phones EMR and the effect of clove bud oil as a powerful antioxidant in protecting the parotid gland against the ROS produced by the mobile phones EMR.

Materials and Methods

The experiment was conducted in the animal house of the Faculty of Medicine, Cairo University, Egypt, according to the guidelines of ethics on animal's experimentation approved by the Animal Use Committee of Cairo University (Approval No: CU/III/F/51/18).

The study design

Study design: (*In vitro* animal randomized controlled trials).

Twelve adult male albino rats were randomly distributed into two groups. Group (A) consisted of six rats as positive control group exposed to the mobile phone EMR and Group (B) consisted of six rats as experimental group exposed to EMR followed by administration of clove bud oil as a treatment for 30 successive days.

After the experiment was completed, the animals were anesthetized with ketamine HCL 50 mg/kg then intracardiac perfusion with 4% formaldehyde for partial fixation of the specimen was given. The parotid salivary glands were dissected for histological and histochemical examination.

The clove bud oil

The clove bud oil was purchased from special outlets of the Ministry of Agriculture in Dokki, Egypt. The oil concentration was 1%, and it was given orally to the rats in a dose of 5 mg/kg/BW/rat following their exposure to the mobile phones EMR for 30 successive days.[10]

Mobile phone exposure protocol

The rats were exposed to EMR emitted from commercially available cellular phone brand energizer (900 MHz frequency, 2 W peak power, average power density of 0.02 Mw/cm2 at specific absorption rate of approximately 0.9 W/KG). The cellular phone was placed over the cage of the rats and the cage was surrounded by aluminum foil to focus the waves and limit the EMR to the interior of the cage.[11] The rats were exposed to EMR 4 h/day for 30 successive days.[12]

Histological evaluation

Hematoxylin and eosin (H&E) stain following the standard technique[13] was carried out to assess the histopathological changes.

Immunohistochemical examination

The oxidative stresses and the ROS in the parotid gland tissue were evaluated using myeloperoxidase (MPO) a marker for LPO.[14]

The immunostained sections were examined using light microscope to assess the prevalence of positive cases and the localization of immunostaining within the tissues. Positive expression was demonstrated as brown discoloration of nucleus, membrane, and cytoplasm. Results were reported as weak, moderate, or strong.

Histomorphometric analysis

Area percentage of myeloperoxidase localization by immunohistochemical staining

The immunohistochemical changes in parotid salivary glands were quantitatively analyzed in term of area percentage. Immunoreactivity of MPO was measured by an image analyzer system using the software Leica Quin 500 (Leica Microsystems, Switzerland). The image analyzer is calibrated automatically to convert the measurement units produced by the image analyzer program into actual micrometer units.

Area percentage was measured in a standard measuring frame in five fields in each group using magnification (400) by light microscopy transferred to the screen. The areas showing MPO positive brown immune staining were chosen for evaluation, regardless the intensity of staining. These areas were masked by a red binary color to be measured by the computer system. Mean value and standard deviation were obtained for each specimen.

The diameter and number of the serous acini

The diameter and number of the serous acini per fields were measured from the H & E stained slides, the image analysis was performed using Leica QWIN V3 image analyzer computer system. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. To determine the number of normal acini/field, we counted the normal

acini in one field. For each group, we used five representative photographs of H&E stained sections for analysis.

Quantitative real time PCR

Quantitative real time PCR was used to measure expression of nuclear factor kappa beta (NF-κB) in the parotid gland tissue.

Total RNA was isolated using Qiagen tissue extraction kit (Qiagen, USA) according to instructions of manufacture. The total RNA (0.5–2 μg) was used for cDNA conversion using high capacity cDNA reverse transcription kit (Fermentas, USA). Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The relative quantitation was calculated according to Applied Biosystem software.

Statistical methods

Data were coded and entered using the statistical package SPSS version 22. Data were summarized using mean and standard deviation. Comparisons between the two groups were done using paired *t*-test. Correlations between quantitative variables were done using Pearson correlation coefficient.[15]

Results

Clinical observation

On daily observation of the albino rats, there were no apparent changes in their daily normal activity.

Histological examination

The histological examination of the parotid gland of Group (A) revealed loss of the normal architecture of the gland manifested in both the parenchyma and connective tissue stroma. The connective tissue septa appeared thick, fibrous [Figure 1a] and infiltrated with large number of inflammatory cells, especially around the execratory ducts [Figure 2a]. There was noticeable shrinkage in the serous acini. Roughly circular acini were seen having a normal lining of pyramidal cells surrounding a narrow lumen. Nuclear pleomorphism was also observed [Red arrow Figure 3a]. Numerous intracytoplasmic vacuolization of variable sizes was observed in the acinar cells [Figure 3a]. Excretory ducts were detected in the C.T septa displaying interruption in their epithelium lining.

Histological examination of albino rat's parotid gland tissue of Group (B) revealed nearly normal connective tissue stroma with apparently no distinctive layers of fibrous tissue [Figure 1b] and less chronic inflammatory cells infiltration [Figure 2b]. The terminal secretory portions showed better histological structure closer to the normal, the serous acini cells showed homogenous cytoplasm and a relatively normal shape with regular alignment around a central lumen and intact distinctive boundaries and less signs of degeneration [Figure 3b]. However, sparse cytoplasmic vacuoles were observed in some acini [Figure 3b]. Less nuclear pleomorphism was detected where the nuclei displayed nearly

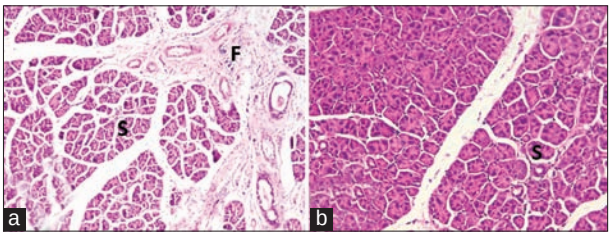

Figure 1: Fibrosis in C.T (F), shrinkage of acini (S) in (a) less atrophy in acini (S) in (b) (hematoxylin and eosin stain, orig. mag. ×10)

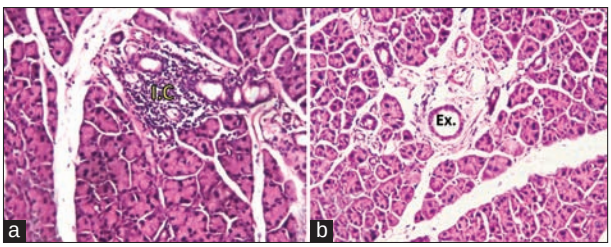

Figure 2: Increased inflammatory cells in C.T (I.C) in (a) as compared to (b) (hematoxylin and eosin stain, orig. mag. ×20)

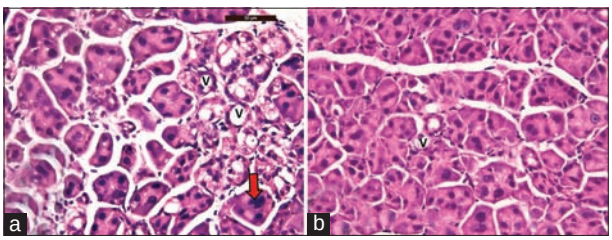

Figure 3: Decreased vacuoles (V) in serous acini in (b) as compared to (a), fig (3a) showing pleomorphic nuclei (red arrow) in acinar cells (hematoxylin and eosin. stain, orig. mag. ×20)

regular shape, size, and chromatin density. The duct system showed almost normal architecture with regular cell lining, the ductal cells were continuous, regular with no significant change in cell shape and size [Figure 2b].

Immunohistochemical examination

The parotid gland of the albino rats of Group A showed strong MPO immunoreactivity in the cytoplasm of the serous acini, excretory duct lining cells, and endothelial lining of the blood vessels [Figure 4a] while Group B showed moderate MPO immunoreactivity in the cytoplasm of the serous acini, striated ducts, excretory ducts, and endothelial lining of blood vessels [Figure 4b]. The immunoreactivity for MPO was negative in all the nuclei of all the parenchymal tissue.

Histomorphometric analysis

Area percentage of myeloperoxidase localization by immunohistochemistry

Comparing the intracellular (cytoplasmic) component of MPO in the acinar portions and in the duct system of the parotid gland

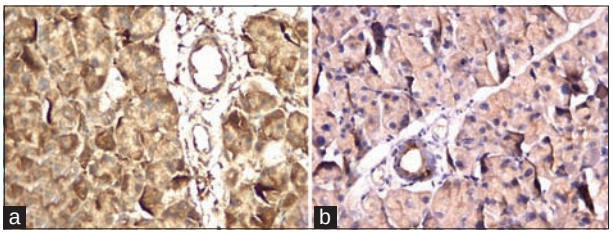

Figure 4: Increased myeloperoxidase immunoreactivity in (a) as compared to (b) (DAB, orig. mag. ×40)

of albino rats among the studied groups revealed a significant reduction in the MPO immunostaining intensity in the cytoplasm of parenchymal elements of Group (B) as compared to that of Group (A), where $P < 0.05$ was considered [Table 1].

Number of serous acini per field

Data of quantitative histomorphometric measurements of the number of serous acini per field were measured and the mean of the number of serous acini was calculated. Comparing the number of acini in Group (A) (mean ± SD=20 ± 2.7) with that of Group (B) (mean ± SD = 24 ± 0.6) revealed that there was significant decrease in the number of acini in Group (A) as compared to Group (B), where $P < 0.05$ was considered [Table 1].

The diameter of the serous acini

Data of quantitative histomorphometric measurements of the diameter of serous acini were measured from six histological photomicrographs from each group and the mean of the diameter of serous acini was calculated.

Statistical analysis for the diameter of the acini in Group A and Group B revealed that there was a significant decrease in the diameter of acini in Group (A) than Group (B), where $P < 0.05$ was considered [Table 1].

Reverse transcriptase-polymerase chain reaction for localization of NF-κB gene expression

Statistical analysis for the NF-κB expression in the parotid glands of albino rats in Group A and Group B showed that the mean value of NF-κB in the parotid gland was higher in Group A as compared to Group B and the difference between the two groups was statistically significant since $P < 0.05$ was considered [Table 2].

Discussion

The present study was designed to study the effect of low-frequency EMR on parotid gland morphology and ultrastructure and to investigate the protective role of clove bud oil from these radiations. Despite the presence of several studies on the effect of EMR on the parotid gland associated with higher frequencies,[16,17] there are limited studies on the effect of EMR on structure and function of parotid gland associated with lower frequencies.

Table 1: Histomorphometric data in the studied groups

P.O.C	Group A	Group B	*P*-value
Area percentage of myeloperoxidase	39.4±5.8	18.9±5.4	0.002*
Diameter of serous acini (μm)	23.8±6.17	35.36±11.4	0.001*
Number of serous acini	20±2.7	24±0.6	0.01*

Table 2: NF-κB mean values in the studied groups

P.O.C	Group A	Group B	*P*-value
Mean values of NF-κB expression	4.19±0.36	1.9±0.39	0.001*

NF-κB: Nuclear factor kappa beta

Numerous histopathological changes were observed in the parotid gland of the positive control group exposed to the EMR (Group A) as compared to the experimental group treated with clove bud oil (Group B). The serous acini of Group A showed intracytoplasmic vacuoles of variable sizes. This finding agreed with those of Ghoneim and Arafat.[3] who reported that mobile phones EMR induced intracytoplasmic vacuoles in the acinar cells of parotid gland of rats. Cytoplasmic vacuolizations of acinar cells of the submandibular salivary gland of albino rats were due to the generation of ROS resulting in LPO and damage of cell membranes.[18]

On the other side, there was decrease in the number of intracytoplasmic vacuolization in Group (B) as compared to Group (A), which could be attributed to the antioxidant effect of clove bud oil which decreased the degenerative changes in the terminal secretory portions of the gland. The forementioned result is in agreement with Abdelrahman *et al.*[19] who reported that clove oil treatment reduced the cytoplasmic vacuolization in the liver of rats with induced inflammation due to its strong scavenging activity that neutralized the ROS.

The parotid gland of Group (A) revealed shrinkage of the serous acini with irregular outlines, degeneration of someone, and pleomorphic, darkly stained nuclei were noticed in others. This finding is concomitant with Fathy *et al.*[20] The deleterious effect of the EMR on the acinar cells could be referred to the effect of ROS on the activation of the mitochondrial pathway of cellular apoptosis which occurs by enhancing the release of cytochrome c from mitochondria and induction of apoptosis.[21]

On the other hand, the serous acini of the parotid gland of Group (B) showed better histological structure where the serous acini cells showed relatively normal shape with intact distinctive boundaries and less signs of degeneration. These could be referred to the protective effect of clove bud oil against the ROS induced by the EMR. Nagababu *et al.*[22] explained the mechanism of protection of liver cells from the cell injury by the antioxidant activity of eugenol. They reported that the eugenol inhibits LPO by scavenging the free radicles (iron and hydroxyl radicals) initiated from the mitochondria through its incorporation into mitochondrial membrane and inhibits LPO by acting as chain breaking agent.

The connective tissue dividing the gland into lobes and lobules appeared thick and fibrous especially around the execratory ducts in Group (A). These findings were concomitant with Aydogan

et al.[23] Increased thickness of the connective tissue could be attributed to the activation and proliferation of fibroblasts and myofibroblasts. The release of ROS with the secretion of chemokines and growth factors such as transforming growth factor beta by immune cells during the inflammation phase is known to promote the activation of fibroblast and collagen deposition in fibrosis.[24] Meanwhile, Group (B) displayed connective tissue of nearly normal thickness and density. This finding is in accordance with a study carried out Abdelrahman *et al*.[19] who found that the clove bud extract hindered fibrosis progression in induced liver fibrosis in albino rats by decreasing the fibroblasts' proliferation due to its ability to neutralize ROS and reduce the oxidative stress.

Marked inflammatory cells infiltration in the connective tissue septa especially around the excretory ducts in Group (A) were in accordance with Aydogan *et al*.[23] The signs of inflammation resulting from EMR are considered as an immunologic reaction against these harmful radiations since the exposure to electromagnetic fields may induce inflammation that can result in fibrosis due to the production of ROS and consequently overproduction of mature collagen fibrils.[25]

On the other hand, the connective tissue stroma of the parotid gland of Group (B) displayed less inflammatory cells infiltration where this histological alteration could be attributed to the anti-inflammatory effect of clove bud oil. These findings agreed with Nikoui *et al*. study.[26]

The excretory ducts in Group (A) displayed interruption in their epithelium lining. This finding is mostly related to the oxidative stress effect of EMR on the parotid gland, and it coincides with those of Anan *et al*.[27] who attributed these changes to the accumulation of ROS in duct lining cells. The excretory ducts of the parotid gland of Group (B) had apparently normal cell lining, with nearly no significant change in cells shape and size. This could be due to the protective effect of clove bud oil over the parotid gland structure where the clove bud has the ability to neutralize the ROS induced in the gland due to the harmful mobile phones EMR.

The immunostained sections of the rat's parotid gland of Group (A) showed strong MPO immunoreactivity in the cytoplasm of the serous acini, excretory duct lining cells, intercalated duct lining cells, and endothelial lining of the blood vessels while the immunoreactivity for MPO in all the nuclei of all the parenchymal tissue was negative. On the other hand, the immunohistochemical results of Group (B) showed decrease in the immunoreaction of MPO as compared to Group (A). The above-mentioned results were in agreement with the results published by Chniguir *et al*.[28] who found that the clove bud aqueous extract decreased significantly the activity of MPO in mice with induced lung inflammation as compared to control group.

The histomorphometric measurements of the diameter and number of the serous acini in the current study revealed statistically significant decrease in the diameter and number of the acini in Group (B) as compared to Group (A). These results were concomitant with Ghoneim and Arafat[3] who showed that exposing the parotid gland of albino rats to mobile phones EMR resulted in statistically significant decrease in the diameter of acini and number of acini per field as compared to the control group which was treated with an antioxidant herb.

The biochemical results of the ongoing study showed statistically significant increased levels of NF-κB in the parotid gland tissue in Group (A) as compared to Group (B). Similarly, Salem *et al*.[29] showed that there was obvious immunoexpression of NF-κB in some acini and granular ducts of submandibular salivary glands of rats administrated sofosbuvir drug. They referred the obvious immunoexpression of NF-κB to the increased levels of ROS as a result of the drug administration. As concerning the biochemical results of Group (B), there was a statistically significant decrease in the NF-κB levels in the parotid gland tissue as compared to Group (A). The forementioned data are in accordance with the results of Yeh *et al*.[30] who reported that the eugenol which is one of the constituents of the clove bud oil inhibited the NF-κB signaling pathways, mediating its anti-inflammatory actions.

The current study highlighted the role of the clove bud oil in protecting the parotid gland from the hazardous mobile phones EMR by the neutralization of the ROS induced in the gland tissues. These findings may pave the way toward the future possible therapeutic applications of the clove bud essential oil.

Conclusions

The overall results of the present study clearly evidenced that EMR had deleterious effects on the parotid glands where it induced numerous histopathological changes that were obvious among the parenchyma and the connective tissue and increased the levels of MPO and NF-κB and decreased the diameter and number of the serous acini. The clove bud oil improved the histological features of the parotid gland in the clove bud oil treated group, decreased the expression of the oxidative stress markers like MPO, and decreased the expression of NF-κB, so it proved to be a strong antioxidant and an anti-inflammatory which protected the parotid gland from the harmful effect of EMR.

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