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The Effects of Pulsed and Sinusoidal Electromagnetic Fields on E-cadherin and Type IV Collagen in Gingiva: A Histopathological and Immunohistochemical Study

Wpływ impulsowego i sinusoidalnego pola elektromagnetycznego na E-kadherynę i kolagen typu IV w dziąśle – badanie histopatologiczne i immunohistochemiczne

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. The potential beneficial effects of extremely low frequency pulsed and sinusoidal electromagnetic fields have been shown on many tissues. Gingival epithelium plays an important role in immunosurveillance of the periodontal tissues. The epithelium acts as a mechanical barrier through cell junctions such as E-cadherin.
Objectives. Investigation of the effects of extremely low frequency magnetic fields on gingiva.
Material and Methods. Twenty-seven male Wistar albino rats were used. The rats were divided into three groups: control group (n = 9), SEMF group (n = 9), PEMF group (n = 9). The SEMF and PEMF (pulse time: 25 μsn, pulse frequency: 50 Hz) groups were subjected to 1.5 mT, 50 Hz, exposure 6 h a day, 5 days a week for 28 days in methacrylate boxes. The gingival tissue pieces processed for routine histological and immunohistochemical examination and tissue sections were stained with H–E and Masson trichrome. In addition, E-cadherin and type IV collagen expressions were examined immunohistochemically.
Results. Intraepithelial lymphocytes and proliferation of epithelial cells increased in both electromagnetic field groups. The over-expressions of E-cadherin on gingival epithelium was detected in the PEMF and SEMF groups. The expression level of type IV collagen was not significant between the control and electromagnetic field treated groups, except for a significant increase in the basal cell layer of the PEMF group, as compared to the control and SEMF groups.
Conclusions. PEMF and SEMF have a local pro-inflammatory effect on gingiva, leading to an increase in E-cadherin level but not type IV collagen. Both PEMF and SEMF can be used as a supportive device in the treatment of gingival diseases, especially those which lead to defects in the epithelial barrier (**Adv Clin Exp Med 2013, 22, 2, 245–252**).

Key words: type IV collagen, E-cadherin, histopathology, pulsed and sinusoidal electromagnetic fields.

Streszczenie

Wprowadzenie. Potencjalne korzystne działanie impulsowych sinusoidalnych pól elektromagnetycznych bardzo niskiej częstotliwości wykazano w wielu tkankach. Nabłonek dziąseł odgrywa ważną rolę w dozorze immunologicznym tkanek przyzębia. Nabłonek działa jako bariera mechaniczna w połączeniach komórkowych, takich jak e-kadheryna.
Cel pracy. Zbadanie wpływu pól magnetycznych bardzo niskiej częstotliwości na dziąsła.
Materiał i metody. Do badań włączono 27 szczurów albinosów płci męskiej szczepu Wistar. Szczury podzielono na trzy grupy: grupę kontrolną (n = 9), SEMF (n = 9), PEMF (n = 9). Osobniki w grupach SEMF i PEMF (czas

impulsu: 25 μsn, częstotliwość impulsu: 50 Hz) poddano działaniu pola magnetycznego 1,5 mT, 50 Hz, ekspozycja po 6 godz./dobę, 5 dni w tygodniu przez 28 dni w klatkach z metakrylanu. Tkankę dziąsła przygotowano do rutynowego badania histologicznego i immunohistochemicznego. Skrawki tkanek barwiono HE i wg Massona. Ponadto zbadano immunohistochemicznie ekspresję e-kadheryny i kolagenu typu IV.

Wyniki. Liczba limfocytów śródnabłonkowych i proliferacja komórek nabłonkowych zwiększyły się w obu grupach poddanych wpływowi pola elektromagnetycznego. Nadmierną ekspresję e-kadheryny w nabłonku dziąsła wykryto w grupach PEMF i SEMF. Ekspresja kolagenu typu IV nie była znacząca między grupą kontrolną i grupami poddanymi wpływowi pola elektromagnetycznego, z wyjątkiem znacznego zwiększenia się warstwy komórek podstawnych w grupie PEMF, w porównaniu do grupy kontrolnej i SEMF.

Wnioski. PEMF i SEMF wywierają miejscowe działanie prozapalne na dziąsła, co prowadzi do wzrostu stężenia e-kadheryny, ale nie kolagenu typu IV. Zarówno PEMF, jak i SEMF mogą być stosowane do wspomagania leczenia chorób dziąseł, zwłaszcza takich, które prowadzą do uszkodzenia bariery nabłonka (**Adv Clin Exp Med 2013, 22, 2, 245–252**).

Słowa kluczowe: kolagen typu IV, E-kadheryna, histopatologia, impulsowe i sinusoidalne pola elektromagnetyczne.

There has been an increasing attempt to research the possible roles and effects of extremely low frequency electromagnetic fields (ELF-EMF) on the tissues and organs of the body over the last three decades [1]. The potential effects of ELF-EMF were shown in several earlier studies. These effects are enhancement of DNA synthesis [2], decrease in bone resorption and maintenance of bone mass [3], protein synthesis [4–6] and gap junctional intercellular communication complex [7], and stimulation of nerve regeneration [8]. Most of the clinical studies and applications were performed with a pulsed electromagnetic field (PEMF), hence only little work has been carried out to evaluate the impacts of sinusoidal electromagnetic field (SEMF). The effect of the mechanism of this type of electromagnetic field on tissues was determined to be different from those of PEMF [9]. As Steffensens et al. wrote, "clinical studies in humans have reported enhanced healing and bone reorganization of nonunion fractures and pseudoarthroses by electrical stimulation following long term unsuccessful conventional treatments" [10]. The epithelium acts as a mechanical barrier through cell junctions such as E-cadherin [11, 12]. As Udey declared, "E-cadherin is a calcium-dependant homophilic cell adhesion molecule that helps in cell-cell interaction." In addition, E-cadherin interaction is thought to be important for retention of the Langerhans cells in the epithelial cells [13]. Gingival epithelium plays an important role in immunosurveillance of the periodontal tissues through the presence of immune cells and production of antimicrobial peptides [14]. The expression of E-cadherin has been shown to be indirectly affected by periodontal disease, presumably as a result of proteolytic destruction by putative periodontal pathogens [15, 16]. ELF-EMF application is described as a treatment method with desirable results where the traditional methods fail. Nevertheless, the possible role of EMF on healthy tissues has still not been elucidated. The aim of this study was to investigate the effects of extremely low frequency pulsed and sinusoidal electromagnetic fields on E-cadherin, which mediate intercellular adhesion between the epithelial cells and type IV collagen expressions in gingiva.

Material and Methods

Animal Care and Preparations for Experimental Animals

The experiments were performed on 27 male Wistar albino rats with initial weights of 150–230 g and aged 4 months approximately, obtained from the Medical Science Application and Research Center of the University of Dicle. All of the rats were permitted free access to water and standard pellet food diet (TAVAS Inc., Adana, Turkey) during the experimental period. All of the rats used in this study were divided into three equal groups: control (Cnt) group (n:9), SEMF group (n:9), PEMF group (n:9). The SEMF and PEMF (pulse time: 25 μsn, pulse frequency: 50 Hz) groups were subjected to 1.5 mT, 50 Hz, exposure 6 h a day, 5 days a week for 28 days in methacrylate boxes (43 × 42 × 15 cm). The animals were kept in a 14/10h light/dark environment at a constant temperature of 22 ± 3°C, and 45 ± 10% humidity.

Generation and Application of Magnetic Fields

The EMF was generated in a device designed and used as noted in an earlier publication, which had two pairs of Helmholtz coils 70 cm in diameter in a Faraday cage (130×65×80 cm) that was earthed to shield it against the electric component (Fig. 1). This magnet was constructed by winding 125 turns of insulated soft copper wire with a diameter of 1.5 mm. Coils were placed vertically as facing one

another. The distance between the coils was 47 cm. An AC current produced by an AC power supply (DAYM, Turkey) was passed through the device. The current in the wires of the energized exposure solenoid was 40 A for 1.5 mT, which resulted in a 50 Hz MF. The MF intensities were measured once per week as 1.5 mT in 15 different points of the methacrylate cage with a Bell 7030 Gauss/Teslameter (F.W. BELL, Inc., SYPRIS, Orlando, Florida, USA), to ensure homogeneity of the field during the course of the experiment by a person who was not involved in the animal experiment. The magnetic field measurements showed that, under the conditions of the experiment, the magnetic field exposure system produced a stable flux density of 1.5 mT and stable frequency of 50 Hz with negligible harmonics and no transients. No temperature differences were observed between exposure and cages during the exposure [17]. For the control, nothing was applied to the rats in this group, and they completed their life cycle in the cage during the study period. The rats were free in a methacrylate cage inside the coils.

Tissue Preparation for Histopathological Examination

A gingival tissue specimen was obtained from the same region of the gingiva after high dose Ketamine HCl (Ketalar, Pfizer) sacrification. On excision, tissues were fixed in 10% buffered formalin for 16 hours and then washed overnight. They were dehydrated with graded alcohol series primarily at 30% absolute ethanol, and next embedded into paraffin. All the sections (histological and immunohistochemical) were evaluated and photographed by using a light microscope (Eclipse i80, Nikon, Japan).

Histological Examination

The paraffin blocks were cut into 5 μm sections and stained with Hematoxylin–Eosin (H-E) and Masson Trichrome. All layers of the epithelium and underlying connective tissue were evaluated histopathologically under light microscope.

Immunohistochemistry

The tissues were put into a formalin solution for fixation and then embedded in paraffin wax. Then they were cut into 4–6 μm sections on positively charged glass slides. Sections were deparaffinized with xylene, followed by immersion in graded alcohol for dehydration and incubation with EDTA (pH:8.0, Merck, Germany) for 5+4+3 minutes in a microwave oven (750 Watt) for antigen retrieval. Next, sections were incubated for 20 minutes in 3% H_2O_2/Methanol to block endogenous peroxidase activity, then rinsed in phosphate-buffered saline (PBS) for 5 min three times. The sections were later incubated with a blocking solution (normal goat serum, Invitrogen, Carlsbad, CA). Slides were then incubated overnight with primary antibodies, E-cadherin (Santa Cruz, 1/100, mouse monoclonal) and type IV collagen (Abcam, 1/500, rabbit monoclonal). After washing in PBS, the sections were treated with labeled-streptavidin kits (Invitrogen, Carlsbad, CA). The reaction was visualized by incubating the sections for 7 min in a 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit, Invitrogen, Carlsbad, CA). Finally, the sections were counterstained with Hematoxylin (Sigma) and covered. Negative control was obtained by the omission of primary antibodies that were replaced with PBS. E-cadherin and Type IV collagen staining status was identified as either negative or positive. Immunohistochemistry positive staining was defined as the presence of a brown color detection chromogen (DAB) on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or plasma membrane of the cells and assessed by light microscope. The stain intensity and proportion of immunopositive cells were also assessed by light microscope. Intensity of staining was graded on a scale of 0–4, according to the following assessment: 0, no detectable staining; 1, weak staining; 2, moderate staining; 3, strong staining; 4, very strong staining. Immunostained slides were blindly evaluated under light microscope.

Statistical Analyses

Statistical analyses were performed with the Statistical Package for the Social Sciences for Windows (version 15.0, SPSS Inc., Chicago, IL, USA). The Mann-Whitney *U* test was used for the statistics as indicated, and the results were expressed as mean ±SD. A p value ≤ 0.05 was considered significant.

Results

Histopathological

The microscopic features were observed as having a normal appearance in control group sections. There were normal characteristic features seen of stratified squamous epithelial cells with keratinized, and in some parakeratinized areas. A few numbers of intraepithelial lymphocytes were observed in this group of sections. Basal membrane

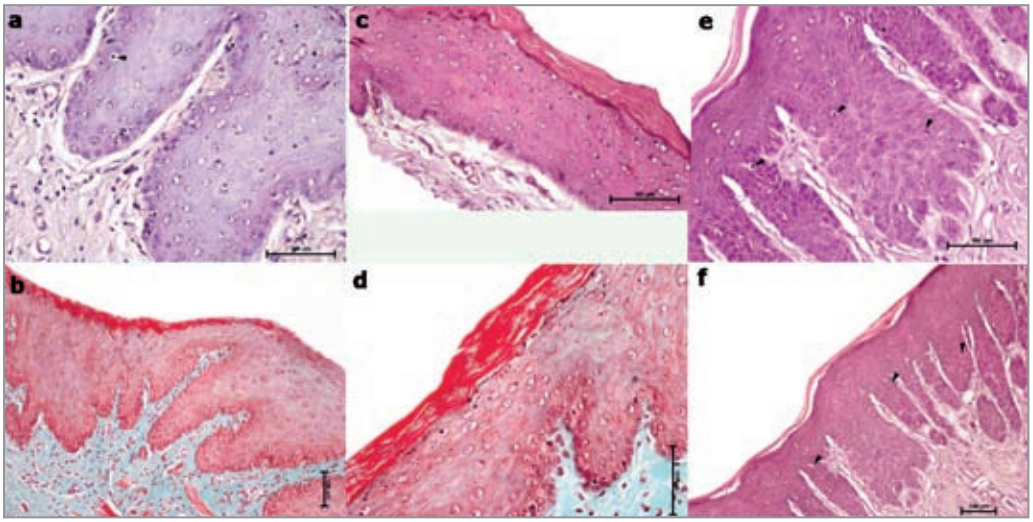

Fig. 1. The representative sections for Masson trichrome and Hematoxylin-Eosin staining in all groups. Normal histological features of gingiva were observed in sections of control groups (a, b). The proliferation of epithelial cells was observed in the PEMF and SEMF groups, not in the control group. The number of rete pegs was similar in both EMF groups (c, d). Intraepithelial lymphocytes were increased in the PEMF group as compared to both the control and SEMF groups (e, f). Arrowheads: intraepithelial lymphocytes

Ryc. 1. Reprezentatywne wycinki barwione wg Massona i hematoksyliną i eozyną we wszystkich grupach. Prawidłowe cechy histologiczne dziąsła obserwowano w wycinkach grup kontrolnych (A, B). Proliferację komórek nabłonkowych zaobserwowano w grupach PEMF i SEMF, ale nie w grupie kontrolnej. Liczba wpukleń naskórka była podobna w obu grupach EMF (C, D). Liczba śródnabłonkowych limfocytów była zwiększona w grupie PEMF w porównaniu zarówno z kontrolną, jak i grupą SEMF (E, F). Groty strzałek wskazują śródnabłonkowe limfocyty

and rete pegs had normal appearance in the control group (Fig. 1a–b). The number of rete pegs was determined to have increased in the gingival samples of both the PEMF and SEMF groups. Intraepithelial lymphocytes increased in both EMF groups as compared to the control. However, the increase in lymphocytes in the PEMF group was more than that seen in the SEMF group. Proliferation of epithelial cells was observed in the basal cell layer in the PEMF and SEMF groups but not in the control group. The difference of proliferation was not significant as compared to each other (Table 1). The beneath of the epithelium, connective tissue, exhibited a variable number of fibroblasts, typical collagen bundles and blood vessels. Inflammatory infiltrates were not existent in all groups (Fig. 1c–f).

Immunohistochemical

To determine the protein expression changes of E-cadherin and type IV collagen induced by an extremely low frequency pulsed electromagnetic field and a sinusoidal electromagnetic field, the authors detected these protein expressions by immunohistochemical assay. A low level of E-cadherin expression was observed in the epithelium of the gingiva of the control group sections especially in the basal cell layer (Fig. 2a). Overexpressions of E-cadherin on the gingival epithe-

Table 1. Histopathological findings in the different study groups

Tabela 1. Wyniki badań histopatologicznych w poszczególnych grupach

	Control (Grupa kontrolna)	SEMF	PEMF	p
Proliferation of epithelial cells (Proliferacja komórek nabłonka)	–	++	++	<0.05
Intraepithelial lymphocytes (Limfocyty śródnabłonkowe)	+	++	+++	<0.05
Elongation of rete pegs (Wydłużenie wpukleń naskórka)	+	++	++	<0.05
Fibroblast (Fibroblast)	+	+	+	>0.05
Collagen bundles (Wiązki kolagenu)	+	+	+	>0.05
Blood vessels (Naczynia krwionośne)	+	+	+	>0.05

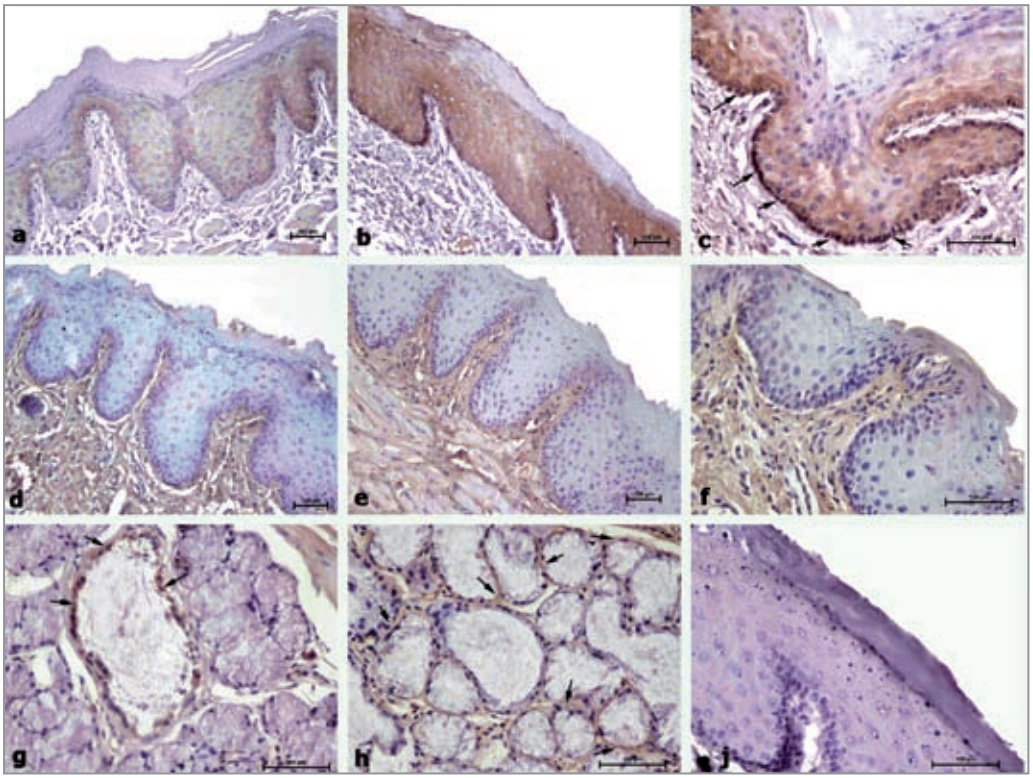

Fig. 2. The representative sections of immunohistochemistry for E-cad and Type IV collagen in all groups. Tissue expressing E-cad and type IV collagen appeared brown in color. The minimal staining for E-cadherin is found in the epithelium of the control group sections (d). Intense staining of E-cadherin was observed in the epithelium of both SEMF and PEMF group sections. E-cadherin expression was observed in the epithelium of ducts of the mucous glands in MF treated groups also to a moderate degree. Expression of type IV collagen was mainly observed in connective tissue, basement membranes of both ducts, epithelia and basal cell layer gingival epithelium (d–j). Arrows: expression of E cadherin and Type IV collagen

Ryc. 2. Reprezentatywne wycinki immunohistochemiczne e-kadheryny i kolagenu typu IV we wszystkich grupach. Ekspresję tkankową e-kadheryny i kolagenu typu IV uwidoczniono w kolorze brązowym. Minimalne barwienie E-kadheryny zanotowano w nabłonku wycinka grupy kontrolnej (d). Intensywne barwienie E-kadheryny obserwowano w nabłonku zarówno w wycinku SEMF, jak i PEMF. Ekspresję E-kadheryny obserwowano w nabłonku gruczołów śluzowych w grupach leczonych MF również w umiarkowanym stopniu. Ekspresję kolagenu typu IV obserwowano głównie w tkance łącznej, błonach podstawnych obu przewodów i warstwie podstawnej nabłonka dziąsłowego (d–j). Strzałki wskazują ekspresję E-kadheryny i kolagenu typu IV

lium was detected in the PEMF and SEMF groups as compared to the control group (Fig. 2b–c). The difference of expression levels in the epithelium of gingiva between the PEMF and SEMF groups was not significant (Table 2). In addition, expression of E-cadherin was detected in some cells of the ducts of mucous glands (Fig. 2g). Type IV collagen expression was observed beneath the epithelium in the connective tissue layer, especially in collagen bundles and the basal membrane of the basal cell layer of the epithelium as well as the basal membrane beneath the cells composed of mucous

Table 2. Expression patterns of E-cadherin

Tabela 2. Wzory ekspresji e-kadheryny

E-cadherin (E-kadheryna)	Control (Grupa kontrolna)	SEMF	PEMF	p
Epithelium (Nabłonek)	+	++	++	<0.05
Basal cell layer (Warstwa podstawna)	+	++	+++	<0.05
Endothelial cells (Komórki śródbłonka)	+	+	+	>0.05
Epithelial cells of ducts (Komórki nabłonkowe kanałów)	+	+	+	>0.05

Table 3. Expression patterns of type IV collagen

Tabela 3. Wzory ekspresji kolagenu typu IV

Type IV collagen (Kolagen typu IV)	Control (Grupa kontrolna)	SEMF	PEMF	p
Collagen bundles (Wiązki kolagenu)	+	+	+	>0.05
Basement membranes of basal cell layer (Błony podstawnej warstwy komórek)	+	+	++	<0.05
Basement membrane of ducts epithelium (Błony podstawne nabłonka kanałów)	+	+	+	>0.05

acini (Fig. 3d–f, h). The expression level of type IV collagen was not significant between the control and EMF treated groups except for a significant increase in basement membranes of the basal cell layer of the PEMF group (Table 3), as compared to the other groups (control and SEMF).

Discussion

The authors investigated the effects of an extremely low frequency pulsed electromagnetic field and sinusoidal electromagnetic field on the gingiva of healthy rats. Two of the main features of epithelial tissues were determined in the organisms. Firstly, cell-cell was closely opposed and attached via specialized junctions. The other was, epithelial cells produced the basement membrane which lies on and separates it from underlying connective tissue. Both features contribute to the barrier function of epithelial tissue [18]. Gingival tissue is constantly subject to mechanical and bacterial aggression. In response to specific antigens or stimuli, inflammatory cells migrate and come into contact in localized areas where they phagocyte the bacteria or damaged tissues. Cellular components of the innate system present in the gingival epithelium include cells such as dendritic cells and neutrophils [19].

Exposure to EMF has not affected their systemic hematologic parameters [20, 21]. However, decreases in the number of white cells were observed in rats exposed to high intensity electrical fields [22]. In addition, Ongaro et al. reported that EMFs have anti-inflammatory effects on osteoarthritis synovial fibroblasts cells by modulating inflammatory and anti-inflammatory parameters [23]. The authors found that no inflammatory infiltration in the connective tissue of gingiva was induced with EMF in the present study. Only slightly increased intraepithelial lymphocytes were found in EFM-treated groups as compared to the control group.

Collagen is a protein and synthesized by fibroblasts, chondroblasts, osteoblasts and other cells. The molecular configuration of collagen fibers confers to them a tensile strength greater than that of steel. Consequently, collagen imparts a unique combination of flexibility and strength to the tissue where it lies. Type IV collagen bundles branch between type I collagen bundles and are continuous with those of the basement membrane and blood vessel walls [18].

The effects of electrical stimulation on periodontal tissue obtained from dogs with bony defects have been investigated. Histopathological examination of this study showed that connective tissue was organized as prominent collagen fiber bundles. In the present study, the authors observed that in the components of the connective tissue, including collagen bundles and fibroblast cells, the underlying epithelium was not impaired from both EMF-treated groups as compared to the control group. In addition, expression of collagen type IV was not significantly different between the groups. Moderate expression levels were observed in connective tissue control and EMFs [24].

The effects of PEMF on increased cell proliferation have previously been shown. In this study, the authors also observed a proliferation of cells in the PEMF and SEMF groups, especially in the basal cell layer of gingival epithelium [25].

E-cadherin is a calcium-dependant homophilic cell adhesion molecule that contributes to cell-cell interaction. The role of E-cadherin is not only attributed to cell–cell adhesion, but also regulates proliferation, differentiation and polarization of epithelial cells [26]. The epithelium acts as a mechanical barrier through cell junctions, such as E-cadherin [11, 12]. E-cadherin interaction is also thought to be important for retention of the Langerhans cells on the epithelial cells [13]. The high expression level of E-cadherin can be an appreciable indicator of the strong barrier function of gingival epithelium [27]. The breakdown of the junctional complex between epithelial cells

was responsible for the destruction of the barrier function of epithelial cells [28, 29]. The authors observed that E-cadherin expression was significantly increased in both PEMF and SEMF groups in the present study.

The authors concluded that extremely low frequency pulsed and sinusoidal electromagnetic fields have a local pro-inflammatory effect on the gingiva of rats. Additionally, both electromagnetic fields increase E-cadherin level and do not change type IV collagen level immunohistochemically. It was concluded that further clinical and molecular studies should be performed to elucidate the effects of electromagnetic fields on gingiva.

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