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ORIGINAL PAPER

Effects of 900 MHz Radiofrequency Radiation on Skin Hydroxyproline Contents

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Abstract The present study aimed to investigate the possible effect of pulse-modulated radiofrequency radiation (RFR) on rat skin hydroxyproline content, since skin is the first target of external electromagnetic fields. Skin hydroxyproline content was measured using liquid chromatography mass spectrometer method. Two months old male wistar rats were exposed to a 900 MHz pulse-modulated RFR at an average whole body specific absorption rate (SAR) of 1.35 W/kg for 20 min/day for 3 weeks. The radiofrequency (RF) signals were pulse modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8 (pulse width 0.576 ms). A skin biopsy was taken at the upper part of the abdominal costa after the exposure. The data indicated that whole body exposure to a pulse-modulated RF radiation that is similar to that emitted by the global system for mobile communications (GSM) mobile phones caused a statistically significant increase in the skin hydroxyproline level ($p = 0.049$, Mann–Whitney U test). Under our experimental conditions, at a SAR less than the International Commission on Non-Ionizing Radiation Protection safety limit recommendation, there was evidence that GSM signals could alter hydroxyproline concentration in the rat skin.

Keywords Radiofrequency radiation · 900 MHz · Skin · Hydroxyproline · Collagen · LC MS/MS

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Abbreviations

RFR	Radiofrequency radiation
LC MS/MS	Liquid chromatography mass spectrometer
SAR	Specific absorption rate
RF	Radiofrequency
GSM	Global system for mobile communications
ICNIRP	International commission on non-ionizing radiation protection
EMR	Electromagnetic radiation
ELF	Extremely low frequency
APCI	Atmospheric pressure chemical ionization

Introduction

The rapid growth of mobile phone communication technology keeps up the debate as whether radiofrequency radiation (RFR) emitted by mobile phones is harmful to humans. While the RFR absorption decreases with an increase in carrier frequency in newer mobile phone generations, from 900 to 2.100 MHz, the absorption pattern has become increasingly closer to the body surface [19]. Therefore, the skin is the most exposed organ to RFR in mobile phone users [28, 32, 34].

Skin tissue is structurally composed of three layers. The epidermis is a 200-μm-thick layer made of distinct layers. These layers are stratum granulosum, stratum spinosum and stratum germinativum, which include keratinocytes and melanocytes. The dermis is 750–800 μm thick. It is rich in collagen fibres and bound water-rich semifluid vascular tissue. The papillary layer of dermis is thin and closely packed with collagen fibres due to interaction between collagen and free water (i.e. bound water). It also contains fibroblasts and merkel cells. The reticular layer of

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dermis contains fatty inclusions from the adipose tissue-rich hypodermis [27].

Collagen, the most important component of animal connective tissue, comprises about one-third of body protein. It is not only the most abundant but also one of the most unusual animal proteins containing the amino acids hydroxyproline and hydroxylysine. In animal tissues, hydroxyproline and hydroxylysine are essentially found only in collagen. For this reason, hydroxyproline has been used as an indicator to determine collagen content. The formation of collagen is of interest not only as an index in the biosynthesis of these unusual amino acids but also as one in protein biosynthesis [38].

There are few studies including contradictory results about radiofrequency (RF) radiation exposure on collagen tissue. Impairment in collagen tissue distribution and separation of collagen bundles in dermis was observed in rats after exposure for 10 days (30 min/day) to a 900 MHz RFR [25]. However, no significant difference was observed in collagen level in the skin of the hairless rat after acute exposure to Global System for Mobile Communications (GSM) 900 or 1,800 MHz radiations at specific absorption rates (SAR) of 2.5 and 5 W/kg [32]. Also, the results of a 12-week chronic study did not demonstrate major histological variations in the skin of hairless rats exposed to a RFR used in mobile telephony (GSM 900 or 1,800) for 2 h/day, 5 days/week and 12 weeks [31]. No significant influence of RFR was reported on filaggrin, collagen and elastin levels after exposure to GSM-900 or GSM-1800 signals for 2 h at 5 W/kg SAR at skin level of the hairless rat [23]. In a study on skin wound in rats, collagenous proteins were found to be lowered after exposure (30 min/day on the first 5 days after wound infliction) to a 42.19-GHz unmodulated RFR, whereas an increase was noted with the same frequency but with a modulation band 200-MHz wide [8].

In another studies performed to asses RF effects on skin, it was showed that exposure to a 900 MHz mobile phone radiation led to significantly higher exocytose in Merkel cells compared with the sham-exposed control [18]. It was reported that human skin cells, after a 1-h exposure to mobile phone electromagnetic radiation (EMR), showed an increased expression of mitogenic signal transduction genes, an increase in cell growth inhibitors and genes controlling apoptosis, and a significant increase in DNA synthesis [26], it was observed that heat shock protein expression either altered or not with RFR exposure of skin, epidermoid, endothelial and fibroblast cells [6, 15, 22, 32]. A significant change in cell proliferation was noted in epithelial cells exposed to signal simulation of GSM of 960 MHz compared to nonexposed cells [42]. Ennamany et al. [9] observed that mobile phone-type EMR (900 MHz) induced a dramatic stress response in the epidermis 'similar to those observed with other stressors, such as acute UV radiation'. [9]. Experimental research by [14] showed that the RFR exposures did not give a statistically significant effect on the development of skin tumours in either transgenic or non-transgenic animals, but tumour development appeared slightly accelerated especially in non-transgenic animals exposed pulsed RFR (SAR of 0.5 Wkg-1, 1.5 h/day, 5 days/week) once it is initiated by exposure to 240 Jm^{-2} UV radiation for 52 weeks [14]. However, another animal study was unable to show a significant effect of chronic exposure to GSM-like signal on tumour latency or cumulative tumour incidence of 7, 12-dimethylbenzanthracene-induced mammary tumours [4]. For the time being, no clear arguments are available to suggest that mobile phone use is associated with an increased risk of skin cancer.

On the other hand, ionizing radiation exposure studies applied on skin showed increases in the collagen type I amount and type I and type III pro-collagens synthesis [3, 20, 33]. Fibrosis is a common side-effect of radiation therapy, resulting from the overproduction or decreased degradation of collagen. Collagen synthesis takes place in fibroblasts. Fibroblasts after radiotherapy treatment enhanced collagen synthesis which is likely to be relevant to the mechanism of radiation fibrosis in the clinical situation [17]. Also, an increase in collagen tissue amount in cultured skin cell lines has been reported with the low frequency electromagnetic field exposure [29]. Collagen syntheses under the effects of a 50-Hz magnetic field of 1, 2 and 3 mT with the exposure duration of 4 h/day for 5 days were investigated.

Previously, studies performed in Gazi Biophysics demonstrated extremely low frequency (ELF) magnetic and electric field effects on skin hydroxyproline level [35, 36]. In this studies, our group reported that while magnetic fields of 1 mT decreased skin hydroxyproline concentrations, 2 and 3 mT increased, and 2 mT was found to be more effective than 3 mT, and skin hydroxyproline level was found to decrease after exposure to a 50-Hz electric field at 1.35 kV/m. Recently, effects of RFR generated by GSM 1800 mobile phones on liver tissue collagen were examined using three different hydroxyproline detection methods by our group. The outcome of the biochemical analysis indicated that RFR did not significantly affect hydroxyproline level [37]. However, the RFR effects on skin hydroxyproline level have not been investigated yet. The aim of the current study was to analyse hydroxyproline content in animal skin exposed to a 900 MHz pulse-modulated RFR for the exposure duration of 20 min daily for 3 weeks. SAR was 1.35 W/kg. For the first time with the present investigation, the hydroxyproline level with RFR exposure was analysed using liquid chromatography mass spectrometer (LC MS/MS) method.

Materials and Methods

Animals

The study was conducted on two-month-old male Wistar rats (weighing 200–300 g). Animals were housed in a room maintained at 22 ± 1 °C and 50 ± 10 % humidity, and under 12-h light–dark cycle (light-on 07:00– 19:00 h). Rats were fed commercial rat chow and given water ad libitum. None of the animals died during the experiment. The investigation was approved by the Local Ethics Committee of the Gazi University Faculty of Medicine. The animals were divided randomly into two groups: sham-exposed group ($n = 10$) and 900 MHz pulse-modulated RFR-exposed group ($n = 10$). The RF-group was exposed to a 900 MHz pulse-modulated RF radiation 20 min/day for 21 days. The sham-exposed group rats were subjected to the same experimental procedure of the RF-exposed group except that the signal generator was turned off. The rats were sacrificed by decapitation following anesthesia by intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) immediately at the end of the last exposure.

A skin biopsy was done at the end of the experiment at the upper part of abdominal costa. The term 'skin' refers to the epidermis and the papillary and reticular layers of dermis. Hair were carefully removed with a blade before taking the specimens. Samples were stored at −80 °C until analysis.

Exposure System

The exposure system consisted of a RF generator (Agilent Technologies, Santa Clara, CA, USA) that produced 900 MHz RF signals, a power amplifier (Hittite, Chelmsford, MA, USA) that amplified the output power of the RF generator, an arbitrary function generator (Thandar, Cambridgeshire, UK) that applied the pulse modulation input of the RF generator, and a rectangular (20–25 cm) horn antenna (ETS-Lındgren, St Louis, MO, USA) facing upwards. The RF signals were pulse modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8 (pulse width 0.576 ms). 217-Hz pulsed signals were observed and verified using an oscilloscope. Polymethyl methacrylate plastics cage (156 × 20 × 20 cm) housing, the rat was placed symmetrically along the axis which is perpendicular and 10 cm above the centre of the horn antenna. The cage was constantly aerated to avoid the possibility of any increase in temperature inside the cage. To obtain sufficient field intensity, a cage was placed in the near field of the antenna. Electric field measurements were performed along the horn antennas axis using an isotropic probe (Rohde and Schwarz, Munich, Germany) and a handheld spectrum analyser RandS®FSH4 (Rohde and Schwarz, Munich, Germany). The RF environmental background level in the frequency range of 30 MHz to 3 GHz was 0.1–0.22 V/m. At the beginning of exposure, average power density was measured at a reference point which was the mid-point of the bottom of the cage wall facing the horn antenna. The maximum power density was observed along the axis of the antenna, and it decreased uniformly with the distance from the antenna's axis.

SAR is a widely used dosimetric quantity to compare the absorbed energy in different biological tissues. The SAR value was estimated by electric field measurements and calculations in this experiment. SAR was computed using the following equation:

$$\mathrm{SAR} = \sigma/\rho|E_{\mathrm{RMS}^2}|\ [\mathrm{W/kg}],$$

where E_{RMS} is the root mean square value of the electric field (V/m), σ is the mean electrical conductivity of the tissues in siemens/meter (S/m), and ρ is the mass density (kg/m^3) (International Commission on Non-Ionizing Radiation Protection [ICNIRP] 1998; Institute of Electrical and Electronics Engineers/American National Standards Institute [IEEE/ANSI] IEEE C95.1–1991). The rat body was assumed to be an equivalent tissue based on the average of the dielectric properties of the 36 tissues in the rat segmented at Brook Air Force Base. Conductivity (0.87 S/m) and mass density (1,105 kg/m^3) were derived for the equivalent tissue using dielectric properties and mass densities of these tissues. The RF exposure in the experiment resulted in a whole body average SAR of 1.35 W/kg with an E_{RMS} field of 41 V/m. Body temperatures of rats were recorded by rectal measurements prior and after exposure session. The RF exposure resulted in a mean rectal temperature increase of 0.28 °C.

Chemicals

High-performance liquid chromatography grade acetonitrile, acetic acid (glacial) were purchased from Aldrich (Milwaukee, WI), and cis-4-hydroxy-L-proline was purchased from Sigma (St. Louis, MO). The chemicals were supplied from company's distributors in Ankara, Turkey. All solutions were prepared in ultrapure water (18.2 MΩ, Milli-Q system, Millipore, Merck KGaA, Darmstadt, Germany). All chemicals were used as received.

Instrumentation

The quantitative measurements of the analytes were performed by LC–MS/MS system including a UFLC liquid chromatography system (Shimadzu prominence, Japan) coupled to a 4000 QTRAP triple quadrupole tandem mass spectrometer (ABI SCIEX, USA) equipped with an atmospheric pressure chemical ionization (APCI) source using multiple reaction monitoring in positive ion mode. An

Agilent Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm particle size, Agilent Technologies, Wilmington, USA) was used with a flow rate of 200 µL/min at ambient temperature. Isocratic separation was performed with a 0.1 % acetic acid:acetonitrile (90:10, v/v) mobile phase. Aliquots (5 µL) of the standard or diluted samples were injected onto the LC–MS/MS system. The following APCI source parameters were applied during measurements: Capillary 5 kV, source temperature 500 °C, nebulizer gas pressure 60 psi, declustering potential 40 and entrance potential 10. Dwell time for the hydroxyproline was set to 250 ms. The electrospray parameters and precursor-to-product ion transitions were determined by direct infusion of the analyte samples into the mass spectrometer. All quantitative measurements of hydroxyproline were carried out using 132 → 68 m/z precursor-to-product ion transition, avoiding shared 132 → 86 m/z precursor-to-product ion transitions of the hydroxyproline and leucine to prevent false-positive measurements. Collision cell pressure was set to medium using collision energy potential 17 and collision cell exit potential 12 for the 132 → 68 m/z precursor-to-product ion transition during the experiments. Thus, skin hydroxyproline concentrations of RF- and sham-exposed Wistar rats were determined.

Standard Curve and Sample Preparation

A hydroxyproline stock solution, containing 500 mg/mL hydroxyproline in water, was serially diluted in 0.1 % acetic acid to prepare a 4-point standard curve ranging from 0.1 to 5 mg/L.

Skin samples were prepared as described in Kindt et al. [21] study. Then, they were lyophilized, resuspended with 1 mL of distilled water, and frozen at −20 °C until analysis. The reconstituted samples were further diluted at least 200-fold with 0.1 % acetic acid during the analysis to keep their concentrations within the standard calibration range. Calculated hydroxyproline concentrations were later multiplied by a dilution factor and normalized by tissue weight or plasma volume for analysis. The study was performed in a blinded fashion with a sham control.

Data Analysis

Data for each group were expressed as means. Statistical analysis was carried out using a SPSS Software Package for Statistical Analysis (SPSS for Windows, Version 16.0.0, SPSS Inc., USA), and the Mann–Whitney *U* test was applied to test for significance of differences between exposed and sham groups. A difference with p value less than 0.05 was considered to be statistically significant.

Table 1 Hydroxyproline concentrations of each animal in sham and exposed groups as determined by LC–MS/MS (ppm)

Sham	Hyp (ppm)	Exposed	Hyp (ppm)
1	9.803	1	11.273
2	10.647	2	10.648
3	10.026	3	12.393
4	10.769	4	13.313
5	5.394	5	15.538
6	5.899	6	11.932
7	11.219	7	12.600
8	8.442	8	6.550
9	12.563	9	11.194
10	10.242	10	8.720
Mean	7,900	Mean	13,100

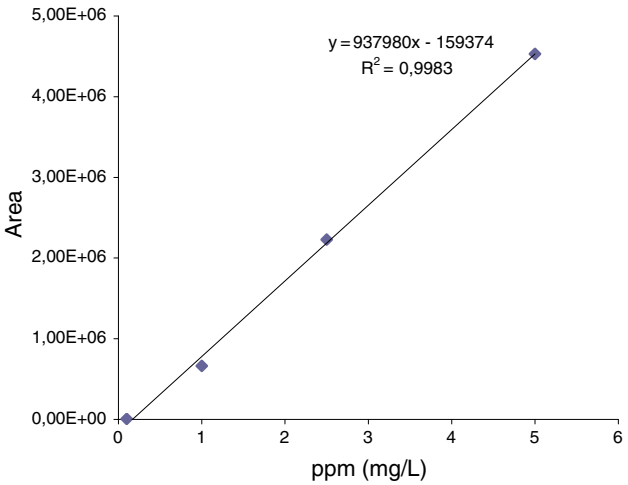

Fig. 1 A typical assay calibration curve with a concentration range of 0.1–5 mg/L. The calibration curve equation for hydroxyproline was $y = 937980x - 159\,374$ with correlation coefficient (R^2) of 0.9983

Results

Table 1 shows hydroxyproline levels in skin samples of RF- and sham-exposed rats. The findings indicated that whole body exposure to pulse-modulated RFR that is similar to that emitted by GSM mobile phones caused a statistically significant increase in hydroxyproline level in the skin ($p = 0.049$). A representative calibration curve is shown in Fig. 1. The calibration curve equation for hydroxyproline was $y = 937980x - 159374$ with correlation coefficient (R^2) of 0.9983.

Discussion

With many emerging technologies going wireless, its health effects on the brain have received the greatest

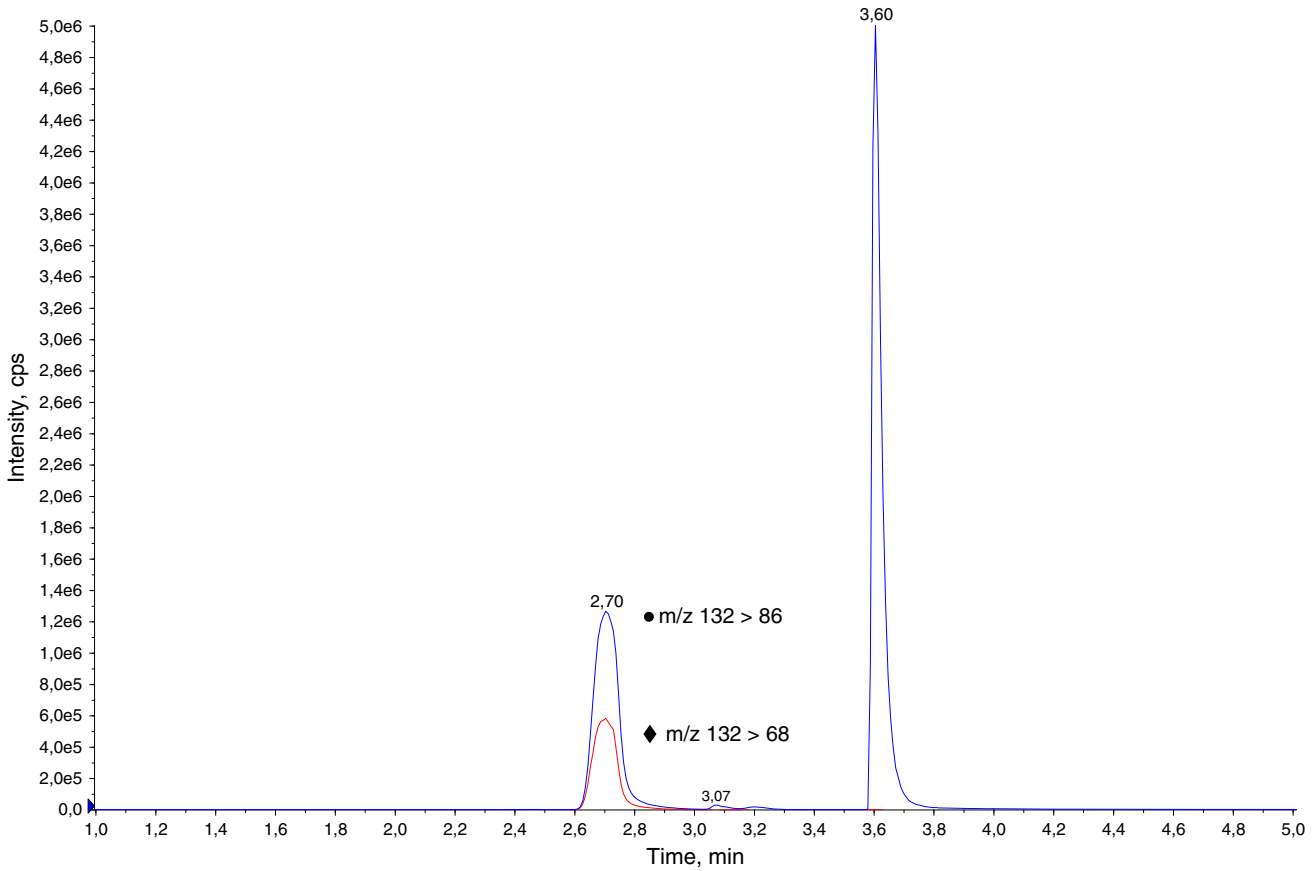

Fig. 2 Representative chromatogram of hydroxyproline. All the quantitative measurements of hydroxyproline (*red line*) were carried out using 132 → 68 m/z precursor-to-product ion transition of the hydroxyproline using MRM mode in LC–MS/MS avoiding similar precursor-to-product ion transitions (132 → 86 m/z) of the hydroxyproline and leucine (*blue line*) to prevent false-positive measurements (Color figure online)

scientific and media attention. However, for electromagnetic radiation to influence the structure and function of the brain, and also other organs, it must first pass through the skin. The skin is our first critical line of defense against all environmental factors, including EMR. Since RFR absorption is mostly superficial; an inevitable result is that it affects firstly the skin. Based on this fact, previously, we have investigated RFR (900 MHz) effect on human hair root cells, and a significant increase in DNA single-strand breaks was observed in cells of hair roots located around the ear which was used for phone calls [41].

In the present study, effects of RFR generated by GSM-900 mobile phones on skin collagen and hydroxyproline formation were examined using LC–MS/MS detection method. The outcome of analysis pointed out that 900 MHz pulse-modulated RFR at a SAR of 1.35 Watt/kg for 20 min/day for 3 weeks significantly increase rat skin hydroxyproline level ($p = 0.049$, Mann–Whitney U test). Since this is a pioneer study on the effect of mobile phone radiation on skin hydroxyproline level, using highly specific method for the determination of hydroxyproline was needed to ensure validity. Although hydroxyproline has been determined with numerous procedures and modifications over the last 50 years such as high pressure liquid chromatography [11], gas chromatography, capillary electrophoresis [39] methods [40, 43], two primary reasons, the selectivity and sensitivity, have blocked efforts towards its convenient analysis. Stegemann et al. [40] and Woessner [43] methods were applied earlier by our group to analyse hydroxyproline level in rats under ELF exposure [35, 36]. In another study, Stegemann- Stalder, Jamall- Finell and ISO 3496 were chosen as biochemical methods of liver tissue hydroxyproline level determination [37]. This paper used LC–MS/MS as a sensitive, selective and reliable tool for quantification of especially hydroxyproline. With the capability of distinguishing differences in the characteristic precursor and product mass transitions of each analyte, tandem quadrapole mass spectrometry imparts a degree of specificity that classical chromatography cannot routinely achieve [21].

The most important challenge for the hydroxyproline quantitation is arisen from the existence of the leucine due to isobaric nature of these molecules. Thus, following only 132 m/z ion signal in ordinary LC–MS experiments can

cause false-positive quantitative measurement about hydroxyproline level. However, different fragment ions of the isobaric species could be generated by MS/MS methods such as CID (collisionally induced dissociation) by adjusting the desired collision energy level of the inert gas molecules (i.e. N_2) and could be followed using MRM (multiple reaction monitoring) method in the LC–MS/MS instruments. Therefore, all the quantitative measurements of hydroxyproline were carried out using 132 → 68 m/z precursor-to-product ion transition of the hydroxyproline using MRM mode in LC–MS/MS, avoiding similar precursor-to-product ion transitions (132 → 86 m/z) of the hydroxyproline and leucine to prevent false-positive measurements. A representative chromatogram obtained by product ion scans of hydroxyproline and leucine species is given in Fig. 2. Subsequent measurements of the samples in LC–MS/MS demonstrated that the amount of hydroxyproline was increased by RFR on skin collagen.

In the present study, we investigated RFR effect on animal skin hydroxyproline level, the indicator of collagen metabolism. Scientists have been interested in collagen for many years because it has piezoelectric characteristics and could be affected by external and/or internal natural electromagnetic fields because of its electrical charge. Most previous research on the effects of electromagnetic radiation on collagen in several tissues was on electric current, static and ELF electromagnetic fields [1, 2, 5, 10, 12, 13, 16, 24, 30]. Only few studies have investigated the effects of 900 and 1,800 MHz RFR on collagen. Ozguner and Celik also found a significant effect on collagen in tissues after exposure to RFR [7, 25]. However, there are studies that showed no significant collagen level changes from exposure to RFR [23, 31, 32, 37].

Our results may indicate a probable effect of collagen synthesis as a result of exposure to RFR from mobile phones. However, we also know that it is difficult to extrapolate effects from rodents to humans because the entire body of a rat is exposed while, for a person using a mobile phone, only the small region of the head around the ear that is close to the phone would be most exposed. Nevertheless, the present study demonstrates the interrelationship between hydroxyproline level and RFR and draws attention to the skin and by so doing may stimulate further research on the effects of mobile phones on the skin.

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