Proliferation Effects of 42-kHz Radio Frequency Energy on Human Foreskin Fibroblasts

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Abstract

Radio frequency (RF), which can penetrate the dermis to induce cell responses, has been frequently used in the field of skin regeneration. This study examines the potential of using RF treatment for skin wound healing. Human fibroblasts were exposed to a 42-kHz RF at various intensities for 30 min. The cell cycle progression, cell viability, and c-fos and c-jun gene expressions were evaluated using flow cytometry, an MTT assay, and reverse-transcription polymerase chain reaction after individual exposures. The results show that the DNA synthesis, cell viability, and gene expressions were upregulated by low-level RF, especially at 350 and 450 A/m² of electromagnetic field exposures. Therefore, RF may play a predominant role in inducing cell proliferation through cell cycle progression and c-fos and c-jun mRNA activation. Non-thermal RF may be the major cause of generating the cell response.

Keywords: Radio frequency, Fibroblasts, Bio-effects

1. Introduction

Wound healing is a complex and dynamic process that involves interactions among soluble mediators, blood cells, extracellular matrices (ECMs), and parenchymal cells [1]. Inflammation, tissue formation, and tissue remodeling, which overlap, are essential phases during wound healing. To regenerate wound tissue, dermal fibroblasts are activated by cytokines and released from macrophages to support skin healing [1]. After the bleeding and inflammatory phases, fibroblasts migrate to the wound margins, proliferate, and secrete ECMs, such as proteoglycans and glycosaminoglycans, to provide a scaffold for directing supportive cells to the injury site and to synthesize more ECMs. However, ischemia, infections, fibroblast inactivity, and wound protease imbalances hinder wound healing, as reported in studies on diabetic, burned, and chronic pressure ulcer patients [2,3]. Therefore, developing an effective method for redundancy reduction has become critical.

Physical therapy modalities are widely applied to wound healing enhancement, such as electrical, ultrasonic, and low-level laser stimulations [4]. Electrotheraphy can stimulate fibroblast growth [5], decrease ulcer size [6], and expedite healing time [7]. Although exogenous electric stimulation assists in the healing process, the current density and voltage distributions are unstable during in vivo applications [8]. Ultrasound (US), which is a type of mechanical stimulation, has longitudinal waves that are used for diagnosis and therapy. Research has suggested that US might facilitate chronic wound regeneration in vivo [9], regulate fibroblastic proliferation [10], and induce ECM deposition [11]. Its noninvasiveness and safety make US one of the most acceptable approaches in clinical applications. However, a medium is necessary for energy propagation, such as water or a coupling gel, and thus there is a risk of infection at the medium contact site. Low-level laser treatment is another promising method for facilitating wound healing [12]. However, the machinery is expensive and the outcomes are controversial. Although these treatments can benefit cell responses, some obstacles must be overcome. Therefore, an effective modality for accelerating wound healing is necessary.

Radio frequency (RF) is one of the most innovative treatments for chronic wound healing and skin regeneration. It has been shown to reduce skin flaccidity, wrinkles, and cutaneous aging [13]. In addition, RF has demonstrated the ability to upregulate human dermal cell proliferation [14,15] and activate expressions of genes and enzymes [16-19]. Moreover, RF generates a non-ionizing electromagnetic field, considered a safe modality for mammalian, and has been used in both medicine and communication depending on the frequency spectrum. For example, the spectrum between 3 and 30 GHz is within the microwave band; the range of 30 to 300 MHz is used for FM radio broadcasts and land mobile stations (emergency and military). Furthermore, RF treatment is non-contact and noninvasive [20-22]. RF may play a
therapeutic role in inducing cell responses and influencing physiological alterations.

The bio-effects of RF are inconclusive [17,23-27]. Particular studies have shown that RF might regulate the behavior of human skin fibroblasts by enhancing DNA synthesis, inducing intracellular mitogenic second messenger formation [17], increasing the expression of comet tail factors, and accelerating centromere-negative micronuclei with time dependence [26]. In addition, a continuous RF wave at 864 MHz significantly upregulates the growth of V79 cells with a specific absorption rate (SAR) of 0.08 W/kg and a 72-h exposure [24]. However, other researchers have argued that RF can induce either harmful effects or have no effect on mammal cells [23,25,27]. Previous study proved that the alkaline comet and cytokinesis-block micronucleus assay of human dermal fibroblasts have no changes after 900-MHz RF treatment for 1 h [25]. Other study reported that human amniotic cells exposed to 900-MHz RF for 24 h at SAR values of 0.25, 1, 2, and 4 W/kg had no significant differences in the aneuploidy rate of chromosomes 11 and 17 [23]. However, a previous study indicated that RF can induce harmful effects on mammal cells [27]. This lack of consensus on the effects of RF on cell responses is probably due to the various RF frequencies, intensities, and exposure durations applied in the studies.

To distinguish these variances, RF treatment at a low frequency of 42 kHz was selected in this study to determine the RF energy effects, because of the rate of energy attenuation in low frequency of RF is lower than that in high frequency of RF. In addition, the biosafety and bio-effects of RF have drawn considerable attention recently. The biological responses can influence certain physiological phenomena, such as wound healing. To determine the efficiency of RF, it is hypothesized that RF influences wound healing by regulating cell proliferation. Therefore, this study investigated the bio-effects of RF in terms of human skin fibroblastic proliferation, which is a leading factor in regulating wound healing.

2. Materials and methods

2.1 Cell culture

Human foreskin fibroblasts (Hs68 cell line) were obtained from the Bioresearch Collection and Research Center at the Food Industry Research and Development Institute (FIRDI) (Hsinchu, Taiwan). The cells were cultured in a medium composed of Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, New York, USA), 4 mM of L-glutamine (GIBCO), 1.5 g/L of sodium bicarbonate (GIBCO), and 10% (v/v) fetal bovine serum (FBS) (Biological Industries, Israel) in an incubator at 37 °C with 5% CO₂ (v/v). Fibroblasts, grown in culture dishes (Corning, New York, USA), were digested with 0.05% (v/v) trypsin-EDTA (GIBCO) for 5 min. The cells were resuspended in a fresh medium, after which they were centrifuged at 1000 rpm for 5 min. All experiments were performed by conducting 2 passages of the cell line after being unfrozen.

2.2 RF exposure system and procedure

The RF induction system consisted of an RF generator (GA-15A, Kanwei Machine and Tool Agent Company, Taiwan) connected to a concentric square coil and a temperature control device, as shown in Fig. 1. The output intensity is adjustable via the electric current. In this study, intensities of 150, 250, 350, and 450 A/m² were applied to cells for 30 min, respectively. The temperature of the culture medium were monitored and maintained at 37.0 ± 0.5 °C using a gas-circulating controller, as shown in Fig. 2. After achieving 80% confluence, Hs68 cells were trypsinized, counted, and resuspended in a medium composed of DMEM supplemented with 10% FBS and 1% antibiotics. Aliquots containing 2 x 10⁵ cells/cm² were seeded in a well of a 24-well culture plate, averaged, and cultured in an incubator maintained at 37 °C in a humidified atmosphere with 5% CO₂. After 24 h, the cells were exposed to 42-kHz RF with various intensities for 30 min in a 37 °C environment.

Figure 1. Apparatus for controlling RF exposure to cells. An electromagnetic field is produced by the coil, through which the current passes. A plate is placed above the coil for exposure. All of the materials are in a sealed styrofoam box. The temperature is maintained at 37 °C during stimulation.

Figure 2. Average temperature differences for 0, 150, 250, 350, and 450 A/cm² RF exposure for 30 min.
2.3 Cell response detection

2.3.1 Cell cycle distribution

To characterize the effect of RF on cell cycle progression, the DNA of cells was stained with propidium iodide (PI) (Invitrogen, California, USA). The Hs68 cells were fixed using 75% ethanol for 30 min and then incubated with a staining solution containing 0.1% Triton X-100 (Sigma, Missouri, USA), 10 µg/mL of RNase A (Invitrogen), and 50 µg/mL of PI for 10 min. Subsequently, the life cycle of the stained cells were analyzed using a BD FACS Caliber instrument (Becton Dickinson, California, USA) and CellQuest software. A minimum of 10,000 cells were counted in each sample.

2.3.2 Cell proliferation

For cell viability analysis, the fibroblasts were cultured in a 24-well plate at a concentration of 20,000 cells/cm² with DMEM supplemented with 10% FBS. At 0, 24, 36, and 48 h after RF exposure, the cells were co-cultured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/mL) (Sigma) for 3 h after RF exposure. The succinate dehydrogenase in the mitochondria of the cells was reduced from a yellow MTT solution to an insoluble and purple formazan, specifically in highly activated cells. Finally, the insoluble formazan was dissolved by dimethyl sulfoxide (DMSO) (Sigma) and measured using a spectrophotometer at a wavelength of 570 nm.

2.3.3 Gene expression

For gene expression analysis, total RNA was extracted using a TRizol reagent (Invitrogen) according to the manufacturer’s instructions. 2.5 µg of RNA was subjected to reverse transcription using a SuperScriptIII RT kit (Invitrogen). The polymerase chain reaction amplifications were activated by Tag polymerase, cDNAs, primers, and other salts for 35 cycles. The primers were c-fos (forward): CCTCACTTTTGAGTCCC; c-fos (reverse): CTCCCTCAGCAGTGGCA ATCT; c-jun (forward): GACTGCAAAATGGAACGACC; c-jun (reverse): GTAGTGGTGATGTGCCCATTGC; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward): CAGC ATGCATCTCGACC; GAPDH (reverse): TGGACTGTGGTCATGAGCCC.

2.4 Statistics

The results represent the averages taken from 3 separate experiments. Data analysis and statistical tests were performed using Student’s t-test.

3. Results

3.1 Cell cycle progression after RF exposure

To determine the effects of RF treatment on cell cycle progression, Hs68 cells were cultured in 24-well plates with a serum-free medium, which prevented interference of growth factors and other nutrition during RF exposure. The cells were then exposed to RF for 30 min at various intensities. After 24 h, the cells were collected, fixed, and stained by PI, and then detected using flow cytometry at a wavelength of 530 nm. The results are shown in Fig. 3. The DNA synthesis (S) portions in the RF-treated groups were significantly higher than those in the sham-stimulation group, reaching 1.6, 1.5, 2, and 1.7-fold increase of control at 150, 250, 350, and 450 A/m², respectively. This indicates that the capability of DNA replication might be activated by RF exposure.

![Figure 3](image-url)

**Figure 3.** S phase portion in cell cycle progression. DNA was stained by PI and detected using flow cytometry. Values are given as the mean of 3 samples ± standard deviation. Statistically significant values are designated as *: p < 0.05; **: p < 0.005; ***: p < 0.001.

3.2 Cell proliferation after RF exposure

Enhanced cell cycle progression has been observed in several physiological phenomena, such as cell proliferation, tissue formation, malignant transformation, inflammation, and wound healing [28]. Although the portions of the S phase increase by RF stimulation has been observed above, the cell fate whether entered the mitosis stage or arrested in DNA synthesis stage remains unknown. To determine the effects of cell cycle progression alteration on cells, cell viability was investigated using an MTT assay. The results show that the cell viabilities of the RF-treated groups, particularly those exposed to 450 A/m², were increased significantly compared that of control within 24 h, as shown in Fig. 4. They reached 1.35-fold increase by RF stimulation at certain day; Xs0: the cell number with RF stimulation at certain day; Xcn: the cell number of control group at Day 0; Xs: the cell number with RF stimulation at Day 0; Xc0: the cell number of control group at Day 0. Values are given as the mean of 3 samples ± standard deviation. Statistically significant values are designated as *: p < 0.05; **: p < 0.005; ***: p < 0.001.

![Figure 4](image-url)

**Figure 4.** Viability of Hs68 cells exposed to 42 kHz-RF at various intensities as a function of time. Xs0: the cell number with RF stimulation at certain day; Xc0: the cell number of control group at Day 0; Xs: the cell number with RF stimulation at Day 0; Xc0: the cell number of control group at Day 0. Values are given as the mean of 3 samples ± standard deviation. Statistically significant values are designated as *: p < 0.05; **: p < 0.005; ***: p < 0.001.
increase of control and gradually decreased in the subsequent 24 h. In the 250 and 350 A/m² RF exposure groups, the cell viabilities were 1.18- and 1.25-fold increase of control after 36 h. Consequently, RF at low frequencies induced DNA synthesis and thus cell viability increased.

3.3 c-fos and c-jun gene expressions

Certain RF-sensitive regulators might be activated by RF exposure, leading to an enhancement of DNA synthesis and cell proliferation. It has been speculated that these cell responses are generated by RF signals transmitted to intracellular sites [17,26]. Thus, immediate early gene families of transcription factors c-fos and c-jun were selected to determine the relevant bio-effects of RF. The results show that the c-fos expressions in the RF exposure groups were significantly activated to 3.5-fold increase of control at 150 and 250 A/m², and to 6-fold increase of control at 350 and 450 A/m², as shown in Figs. 5(a) and 5(b). The expression of c-jun was significantly activated by RF at 350 and 450 A/m² groups, to 3- and 2-fold increase of control, respectively. These results suggest that the upregulation of c-fos and c-jun gene expressions may correlate with cell cycle progression and cell proliferation in Hs68 cells exposed to RF.

RF is an innovative method used in wound healing. Several auxiliary treatments have recently been developed to regenerate wounds. Whirlpool, subatmospheric pressure, pulsed lavage, and hyperbaric oxygen therapy have potential in becoming mainstay therapies, despite limitations such as cost, complications, and diversity of effects [29]. In addition, US, electrical stimulation (ES), and RF have been proven to have positive effects on wound healing. However, additional in vitro and in vivo experiments are required to determine the efficiencies of US, ES, and RF in future applications. Low-frequency RF was selected in this study because it is non-contact and noninvasive and lacks side effects. However, the bio-effects of RF remains unclear. Therefore, an apparatus and a procedure were developed for efficiently exploring RF stimulation in vitro to investigate cell responses. Furthermore, RF is expected to be used in chronic wound healing applications [15,30].

The results show that the progression of DNA synthesis was induced after RF exposure. An entire cell cycle consists of four phases, namely Gap 1 (G1), DNA synthesis (S), Gap 2 (G2), and mitosis (M), which are regulated by the coordinated activation of cyclins and cyclin-dependent kinases [31]. For example, cyclin C, cyclin D1, and cyclin E are the checkpoint proteins that determine the transition from the G1 phase to the S phase; cyclin A and cyclin B1 regulate mitotic G2/M transition and mitosis [32]. Certain growth factors and physical stimuli can regulate quiescent cell reentrance into cell cycle progression. RF-exposure-enhanced cell cycle progression was demonstrated in this study. Therefore, RF could be the main cause involving in the regulation of DNA synthesis. Certain studies have shown results that are consistent with those of this study. Cao et al. [33] reported that 27-MHz RF at 25 W/kg altered the cell cycle progression of Chinese hamster ovary cells, which is consistent with our results. However, neither single (837-MHz) nor combined (837- and 1500-MHz) RF modes altered both the cell cycle distributions and the cell cycle regulatory proteins at a SAR of 4 W/kg and 1 h of exposure [34]. The cell-cycle-related genes of HL-60 cells were downregulated when the cells were exposed to 2.45-GHz RF for 2 h [35]. Taken together, variations in RF parameters, such as frequency and duration of stimulation, may lead to such inconsistent results. This study has demonstrated that low-frequency RF appears to have great potential in accelerating cell cycle progression. Further experiments are required to optimize the frequency and duration of RF stimulation in Hs68 cells for cell proliferation enhancement.

The cell proliferation results show that RF may play a vital role in cell proliferation enhancement. Numerous causes can induce increases in cell numbers. First, the electromagnetic fields may directly affect the gene expressions related to cell proliferation in the nucleus and, consequently, increase cell growth. Second, certain RF-sensitive receptors distributed in the cell membrane may be activated by electromagnetic field exposure. Cytoskeletons, such as proteoglycan and F-actin,
translate the signals to induce cell responses. Third, electromagnetic fields can stimulate particular soluble-growth factor secretions in the culture medium. These growth factors can possibly benefit cell proliferation. Finally, RF may cause perturbation of the culture medium, which could accelerate the exchange and metabolism of ions and nutrition. However, additional experiments are required to demonstrate the mechanisms of these potential pathways.

Other studies have shown that RF exposure upregulates cell proliferation, which is consistent with our study [36-38]. With exposure to 72-GHz microwaves in continuous-wave (CW) mode, Candida albicans had a 25% increase in its cell growth rate [37]. Increasing concentration in bacterial cultures exposed to 2.45-GHz CW microwaves and 3.10-GHz pulsed microwaves has been observed [38]. Therefore, non-ionizing exposure enhances cell proliferation, which is consistent with this study. However, certain studies have reported that RF has no effect or negative effect in cell proliferation [34,39,40]. The proliferation of human lens epithelial cells remained unchanged after 1.8-GHz RF exposure [39]. The proliferation of V79 cells exposed to 935-MHz RF was downregulated [40]. Variations in the characteristics of the RF and target cells might be the cause of these conflicting results.

To determine how RF initiate cell proliferation, c-fos and c-jun genes were considered to be potential mediators and selected to determine the outcome of RF exposure. The results show that RF enhanced c-fos and c-jun gene expressions. c-fos, c-jun, and c-myc are a family of immediate early proto-oncogenes, which are mediated by mechanical signals and may initiate cell growth [41,42]. Our results show that both the immediate early gene expressions and cell proliferation were increased by low-frequency RF. In addition, cell growth (24 h after RF exposure) occurred directly after gene expression (2 h after RF exposure), indicating that cell proliferation is a downstream consequence of gene expression. However, the RF mechanisms regulating cell proliferation through immediate early gene pathways are unclear. Additional experiments are necessary to confirm the relations among these cell responses.

Gene expression alterations after RF exposure have been reported since the global system for communication became widespread [16,17,30]. The gene expressions of cell cycle regulators, mitogen-activated protein kinase 3, extracellular signal-regulated kinases 3, and transforming growth factor-β in human skin fibroblasts were shown to be activated by 902.4-MHz RF [17]. Similarly, an increase in c-jun transcript levels of PC12 cells caused by 836.55-MHz RF exposure has been observed [16]. The upregulation of inflammation gene expression has also been confirmed to be induced by 27.12-MHz RF for 30 min exposures [30]. This shows that RF enhances the expression of specific genes. However, numerous studies using various RF fields and cell types have found diverse effects on gene expression [43-46]. For example, the expression of FBJ murine osteosarcoma viral oncogene homolog was not significantly changed when C3H 10T1/2 cells were exposed to RF fields of 847.74-836.55 MHz [45]. c-fos and c-jun mRNA expressions were not affected by 900-MHz electromagnetic field exposures [44], and no significant gene alterations were observed in normal human glial cell lines exposed to 2.45-GHz RF fields [43]. The RF effects are thus inconclusive, and additional studies are required to distinguish the bio-effects in clinical applications.

The enhancement of cell cycle progression, increases in cell proliferation, and inductions of c-fos and c-jun gene expression are expected to be affected by the thermal and non-thermal effects of RF. However, the temperature differences of culture media between the sham and exposure groups were less than ±0.5 °C (Fig. 2), the non-thermal effect appears to be the main cause of inducing Hs68 cell biological responses. Electromagnetic fields consist of electrical and magnetic fields, which may induce different bio-effects. Alternating electrical fields of high frequencies raise water temperature because of the friction of rapidly rotating dipolar molecules in water [47]. However, the temperature did not change during the 42-kHz RF exposure in this study. A previous study has shown that the electrical field does not alter cell concentration at 27 MHz, indicating that electrical fields with lower frequencies do not affect cell viability [37]. Therefore, it appears that magnetic fields may be the primary cause of the induced cell response.

Although RF at 42 kHz was demonstrated to enhance DNA synthesis, cell proliferation, and gene expression, additional experiments are necessary to prove the correlation among cell cycle progression, cell number upregulation, gene expressions, and the magnetic field mechanisms that induce cell responses. The potential bio-effects of RF exposure on the surrounding cells of skin tissue, such as keratinocytes, blood cells, and lymphocytes were unclear in this study. However, studies have shown that RF does not affect lymphocyte proliferation [26,48], apoptosis in peripheral blood mononuclear [49], and protein expression in MCF-7 [50]. Although these results indicate that RF does not alter cell fate, the different cell responses could depend on the RF parameters. Therefore, the bio-effects on surrounding cells must be investigated in future studies to avoid abnormal responses and maintain physiological homeostasis. In addition, the parameters of RF treatments are crucial for future in vitro and in vivo applications.

5. Conclusion

This study investigated Hs68 cell response to 42-kHz RF at various intensities. The results show that the portions of S phase in the cell cycle, cell number, and c-fos and c-jun gene expressions significantly increased when the cells were exposed to RF for 30 min. RF induced c-fos and c-jun gene expressions, resulting in cell cycle progression and an increase in cell numbers. Additional studies are required to quantify the interactions between cell responses and RF exposure in animal models to determine whether these effects regulate wound healing.

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References


