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Effects of Schumann's Resonant Frequencies on *In-Vitro* Normal Human Dermal Fibroblast (NHDF) Cells Using Pulsed Electromagnetic Field (PEMF)

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Abstract: We analyzed the effects of Schumann's resonant frequencies on *in-vitro* normal human dermal fibroblast (NHDF) cells using pulsed electromagnetic field (PEMF) therapy. A Master Oscillator Power Amplifier (MOPA) brand PEMF device was used to generate signals in the radio wave region. Schumann's resonant frequencies from the fundamental frequency up to the sixth harmonic were chosen (Price, C., 2016). These resonant frequencies were grouped and assigned as P2 for the 7.8, 14, 21, 26, 33, 39, and 45 Hz; 7.8, 14, and 21 Hz for P3 set, and; P4 for the 26, 33, 39, and 45 Hz as exposed groups all in 10 and 20 minute exposure times. Three wells were pipetted with NHDF cells for each program in a 24-well plate for each exposed group. Using these exposed groups, we observed tissue growth of NHDF on each well plate by monitoring the micropipette (P200) tip induced wound gap using an inverted phase contrast microscope coupled with an *ImageJ* software. Statistical analyses using one-way analysis of variance (ANOVA) test and F-test were done to estimate the significant effects of PEMF on NHDF cell growth compared to the control group assigned as P1. After 48 hour of incubation period, it was shown that the P2 group with 10 minute exposure time has the highest yield at 97.74% wound gap closure. While P2 and P3 groups with 20 minute exposure time yielded 100% wound gap closure after 48 h incubation period. P4 group for both exposure times yielded the lowest wound gap closure after the 48h incubation period. Exposed groups of P2 and P3 have shown significant effect on the NHDF tissue growth compared to the control group after two days or 48 hour incubation period and thereafter using ANOVA and F-test.

Key Words: Pulsed electromagnetic field; Tissue growth; Schumann's resonant frequencies; Normal human dermal fibroblast cells

1. INTRODUCTION

Cellular interactions from pulsed electromagnetic field (PEMF) therapy have attracted the attention of researchers in regenerative medicine over the past number of decades (Alcaraz, *et al.*, 2017, Madduri, 2018). PEMF therapy is a type of non-invasive, non-thermal electrotherapy that utilizes an active electromagnetic waveform typically delivered through an antenna to treat an area of tissue on a subject. Many different types of PEMF devices have been developed for both research and clinical applications (Gaynor, *et al.*, 2018). Since PEMF therapy has been shown to decrease markers of inflammation, it is also a potential therapeutic method in soft tissue and bone healing environments. Studies have shown that it has been clinically used to improve structural and functional effects in both human and veterinary medicine. Furthermore, the implementation of PEMF therapy for treatment on non-union fractions, post-operative pain and edema, inflammation and arthritis, and chronic wounds, have been approved by the U.S. Food and Drug Administration (FDA) (Huegel, *et al.*, 2018, Wang *et al.*, 2015).

In 2018, Huegel, *et al.* studied the effects of PEMF therapy on rotator cuff tendon-to-bone tears in a rat model. The purpose of this study reflected the influence of both PEMF frequency and exposure time on rotator cuff healing. The investigation showed adult male Sprague-Dawley rats underwent bilateral acute supraspinatus injury and repair following a control and randomized treatment classification groups. The treated groups either received a Physio-Stim (PS)

PEMF therapy or a high-frequency PS PEMF therapy. The research concluded that PEMF treatment increased tendon cell metabolism, sequentially increasing both collagen production and matrix remodeling. PEMF signal characteristics typically differ between applications with regards to waveform type, signal intensity, and treatment duration. Varying parameters, including frequency and treatment duration, have been identified to cause differences in cell and tissue response (Huegel, *et al.*, 2018).

Given the many possible effects of Schumann resonances in the human body, the researchers decided to focus on the effects on a microscopic level—fibroblast cells in a*n in-vitr*o environment is a suitable avenue to perform the study.

In this study, the researchers have built a baseline case that aims to understand the mechanism of Pulsed Electromagnetic Field Therapy (PEMF) and its effects on the metabolic activity of Normal Human Dermal Fibroblast (NHDF) cells under an *in-vitro* environment. In particular, this study focused on the effects of Schumann's resonant frequencies and exposure time in the growth of NHDF cells under low intensity conditions. Master Oscillator Power Amplifier (MOPA) device, a device capable of generating frequencies in the radio wave region, was utilized. Using the *ImageJ* software, the wound gap size of viable NHDF cells in scratch assay platform was measured and analyzed. This study presents a non-invasive approach in handling Human Dermal Fibroblast (HDF) cells that might provide additional insight on possible mechanisms behind the cell-PEMF interaction within Schumann's resonant frequencies. Given this advantage, the reduction of time needed to regenerate these skin cells can minimize possible wound infection in the future.

2. METHODOLOGY

2.1. Flowchart of the Study

The cultured NHDF cells were exposed under low-intensity PEMF. The cells were divided into a control group, labeled as Program 1 (P1), and an exposed group. The exposed group was further categorized into Program 2 (P2), Program 3 (P3), and Program 4 (P4). The researchers considered if a lower or a higher set of frequencies can affect a faster wound closure and cell count on the samples. Hence, the groupings of the frequencies. Each program consists of a 10 min and 20 min exposure triplicates. With a total of 7 plates, all NHDF cell triplicate were monitored during an incubation period of 0 h, 48 h, and 72 h to observe the progress of the wound gap closure. Images were taken and analyzed using *ImageJ*, GraphPad Prism 8, and Microsoft Excel (PHStat 2.0) software to monitor the cell count and determine if there is a significant difference between cell count of the control and the exposed groups. Fig. 2.1 illustrates the flow of the study.

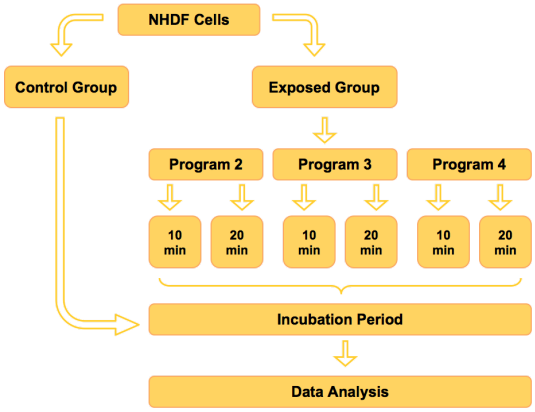

Fig. 2.1. Flowchart of different groups.

2.2. Materials and Equipment

2.2.1. PEMF Device Configuration

The frequencies set for the exposed groups were pre-configured using a desktop computer connected to the MOPA device. The following steps were done to create a custom channel: First press the "Custom" button to begin > Choose the "Group" button to input the channel's frequencies > Click the "Custom" button once more to save all frequencies for the corresponding channel.

2.2.2. Cell Culture

NHDF cells were cultured in a 75 mL T-flask. The cells were suspended in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 4°C for several days. The flask was trypsinized after the removal of the DMEM, allowing the adhered cells to

float. Cells were resuspended by adding 75 mL DMEM before transferring to seven, 24-well plates. The cultured NHDF cells were then transferred into the wells by pipetting 1 µL in triplicates: Program 1 has a triplicate of 1 µL per well and Programs 2, 3, and 4 have 1 µL per well, two sets of triplicates each, where each triplicate were placed on both corners of the well (see Fig. 2.2). The plated cells were then incubated and monitored until they reached 90% to 100% confluency. The confluency determines if the researchers may proceed with the scratch assay.

Fig. 2.2. P2, P3, P4, and P1 well plates arranged respectively. The exposed groups contain two sets of triplicates on each end while the control group contains one set of triplicate. Behind the well plates is the MOPA device.

2.2.3. Scratch Assay

The scratch assay involved a confluent cell monolayer that was scratched vertically using a p200 pipette tip. All cultured NHDF cells of all the programs were scratched subsequently with one another. The open gap that was created by this "wound" was monitored microscopically during the incubation period. Fig 2.3 illustrates a monolayer cell culture with an introduced wound using the p200 pipette tip. The cells were observed under an inverted phase-contrast microscope while images were captured with its camera. As a means of overseeing the progress of cell migration movement, each well plate of the NHDF cell line was taken a picture of in the area of wound gap of all 4 programs, for both 10 min and 20 min exposure times. The cells were observed immediately after exposure (0 h), after two days from the time of exposure (48 h), and after three days from the time of exposure (72 h).

Fig. 2.3. Image of control group P1 monolayer cell with induced wound under the inverted phase-contrast microscope. The image was taken during the 0 h incubation period.

2.3. Data Acquisition

2.3.1. ImageJ Analysis

ImageJ is a software that was used for cell counting. It contains tools allowing users to perform specific actions relative to the study in relative pixel units (RPU). It was used to "count" the cells in the obtained images. It is a free platform easily accessible through https://imagej.nih.gov/ij/download.html. Fig. 2.4 illustrates a raw image and an enhanced image using the *ImageJ* software.

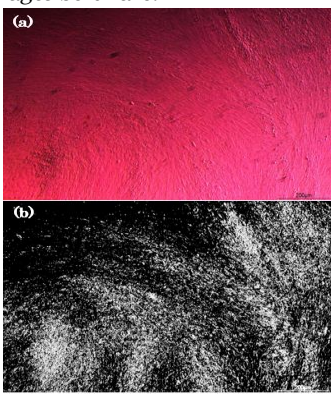

Fig. 2.4. (a) Image of P3 10 min for the 48 h incubation period; (b) The enhanced image of P4 10 min using the *ImageJ* software

To properly "count" the cells, certain enhancements were made on the images. The following steps were performed to make specific enhancements: Process tab > Find Edges > convert to 8-bit type by going to Image > Type > 8-bit. Afterwards, the threshold was adjusted under Image >

Adjust > Threshold. The resulting image was then used to "count" the cells using Analyze > Analyze Particles. A group of parameters were set prior to counting the cells: 1) Size = 0-infinity; 2) Circularity = 0.00-1.00; 3) Show = Outlines.

2.3.2. MRI Wound Healing Tool

Similar to the *ImageJ* software, the MRI Wound Healing Tool can be accessed and downloaded easily. It is a special tool that is incorporated to the *ImageJ* interface to perform its function—in this case, to analyze wound gap closure. Likewise, certain enhancements were made on the images. The selected images were enhanced under Process > Find Edges. The images were also converted into grayscale by clicking on the Image tab > Type > 8-bit. The threshold was adjusted so as to make the outline of the gap clearer by Image > Adjust > Threshold where the upper slider was set to the leftmost part and the lower slider was set according to the researchers' preference. The *m* tool was selected in order to start the measurement on the image. Fig. 2.5 compares the images of before and after enhancing a "wounded" monolayer cell.

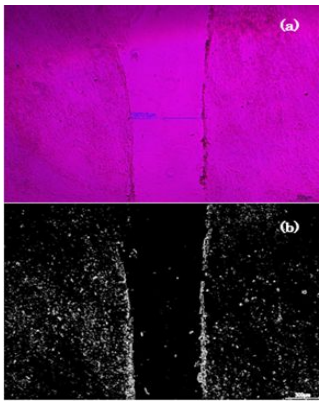

Fig. 2.5. (a) An image of P4 10 min monolayer cell under 0 h incubation period; (b) An enhanced image of P4 10 min under the 0 h incubation period ready to be analyzed using the *m*-tool feature of *ImageJ*

2.3.3. Area Percentage Calculation

One image per program was selected to calculate the percentage of wound gap closure. Eq. (2.1) was used to calculate the area percentage of wound gap closure for both 48 h and 72 h incubation periods.

$$\%Area_{Closure} = \frac{Area_{initial} - Area_{after\ 48h/72h}}{Area_{initial}} \times 100\% \quad \text{(Eq. 2.1)}$$

where:

$Area_{initial}$ = area obtained immediately after the introduction of the wound gap using the p200 pipette tip

$Area_{after\ 48h/72h}$ = area obtained after 48 h/72 h from the time of exposure

2.3.4. Statistical Test using PHStat

The one-way analysis of variance test (ANOVA) was used to determine if there are significant differences on the cell count (obtained through *ImageJ*) of exposed groups as compared to the control group P1. PHStat, an extra tool in Microsoft Excel which can perform statistical analysis, was used to perform these analyses—F-test was used to determine if the distribution of the test samples is widely varied or not prior to testing the one-way ANOVA. The procedure was carried out by arranging the data of P1 and the exposed groups in columns, then under Data > Data Analysis > Anova: Single Factor was selected. The specified test was used to perform individual analysis for all the incubation period groups—one separate test for 0 h, 48 h, and 72 h where the independent factor was the control group P1. The obtained *p*-value was compared to the standard confidence interval of $\alpha = 0.05$: If the *p*-value is lower than the confidence interval, then a significant difference is observed.

3. RESULTS AND DISCUSSION

3.1. Cell Count

According to the tests performed on the groups per incubation period, the cell count and the rate of wound gap closure were obtained. Table 3.1 and Table 3.2 compares the cell count of all the groups per incubation period exposed for 10 min and 20 min respectively.

Table 3.1. Average cell count measured through relative pixel units (RPU) obtained over the 72-h incubation period, exposure time = 10 min

Group	Frequencies (Hz)	Cell count at 0 h (RPU)	Cell count at 48 h (RPU)	Cell count at 72 h (RPU)
P1	Control group	2099.0	3203.2	4912.5
P2	7.8, 14, 21, 26, 33, 39, 45	1847.3	4132.8	6232.5
P3	7.8, 14, 21	2298.7	3402.5	5803.3
P4	26, 33, 39, 45	1837.3	2552.2	5923.2

Table 3.2. Average cell count measured through relative pixel units (RPU) obtained over the 72-h incubation period, exposure time = 20 min

Group	Frequencies (Hz)	Cell count at 0 h (RPU)	Cell count at 48 h (RPU)	Cell count at 72 h (RPU)
P1	Control group	2099.0	3203.2	4912.5
P2	7.8, 14, 21, 26, 33, 39, 45	2153.7	3629.7	6481.0
P3	7.8, 14, 21	1560.7	3709.7	6482.5
P4	26, 33, 39, 45	698.0	2895.5	5627.0

From the tables presented, it was observed that the exposed groups exhibited higher cell count as compared to the control group P1. Only the exposed group P4 was observed to have yielded lower cell count for the 0 h and 48 h incubation period but increased after 72 h relative to P1 from the time of exposure. A possible explanation attributing to a lower cell count for P4 at 0 h could be due to the manual introduction of wounds on the cultured NHDF cells. Since the researchers manually induced a wound using a P200 pipette tip, sizes of the wound gap varies from P1 to P4. Thus, the difference in cell count. After performing a one-way ANOVA test, only P4 20 min obtained a *p*-value lower than 0.05 for 0 h incubation period. However, this data can be negligible as the trend of the cell count of P4 increased throughout the incubation period, following the trend of all the other programs. Furthermore, the effects of the frequencies assigned for P4 may not be immediately observed shortly after exposing the cells. For the 48 h incubation period, only P2 10 min established a significant difference relative to P1. At the end of the incubation period, P2 10 min, P2 20 min, and P3 20 min exhibited significant differences as shown in Fig. 3.1.

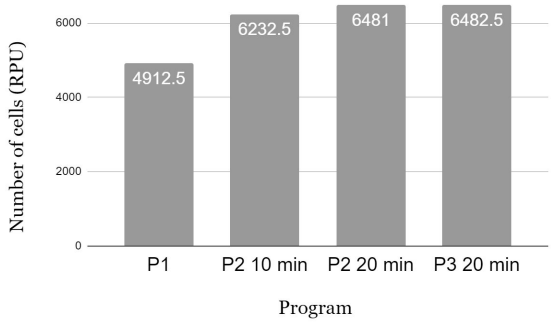

Fig. 3.1. Comparison of the program groups with significant differences relative to P1. As observed from the graph, the values obtained for the cell count of P2 10 min, P2 20 min, and P3 20 min are precise.

3.2. Rate of Wound Gap Closure

It was observed that the exposed groups exhibited a 100% wound gap closure immediately after 48 h from the time of exposure for 20 min exposure time. The control group P1 only acquired 35.02% of wound closure at the same time of observation. Considering that P1 was not exposed under the PEMF, the migration of the fibroblast cells into the wound site was slower as opposed to the exposed groups. Similarly, the exposed groups were observed to have higher rates of wound gap closure as opposed to P1. The only exposed group which yielded a lower rate is P4.

Table 3.3 shows the data obtained for the rate of wound gap closure for both the 10 min and 20 min exposure time obtained using the equations mentioned above.

Table 3.3. Wound area percentage obtained over the course of the study

Group (frequencies)	Exposure (min)	Area 0 h (RPU)	Area 48 h (RPU)	Area 72 h (RPU)	%Area wound closure after 48 h	%Area wound closure after 72 h
P1 (control group)	-	520767	338394	0	35.02%	100%
P2 (7.8, 14, 21, 26, 33, 39, 45)	10	462470	10470	0	97.74%	100%
	20	625591	0	0	100%	100%
P3 (7.8, 14, 21)	10	262921	42130	0	83.98%	100%
	20	546176	0	0	100%	100%
P4 (26, 33, 39, 45)	10	312018	240731	0	22.85%	100%
	20	543589	0	0	100%	100%

4. CONCLUSION

The *in-vitro* study of the effects of PEMF on NHDF cells showed that Schumann's resonance frequencies can help induce faster wound gap closure. To be specific, exposing the cells for 20 minutes is far more effective in closing the wound gap compared to a 10 minute exposure time. When the cells were exposed for 10 minutes, the set of frequencies for P2 had a faster impact to the wound gap closure followed by P3. The variable group P4 yields the least effective in terms of rate of wound gap closure. Statistical analyses showed that certain frequencies effect higher cell count compared to the control group P1. Exposed groups P2 10 and 20 min and P3 20 min were observed to exhibit higher cell count among all groups.

Given the parameters monitored closely by the researchers, P2 10 min was proven to be the most optimal set of frequencies and exposure time in influencing higher cell count and faster wound closure.

5. ACKNOWLEDGEMENT

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