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Study of Ultra-weak CW and Amplitude-Modulated Microwaves effects on stem Cell Proliferation: an Experimental and Hypothetical Approach

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ABSTRACT: Weak microwave radiation (WMR) in our environment has raised health concerns in the public. Among those, communication frequencies are more than ever becoming widespread and their effects need thorough studies. A correct understanding of these effects in-vivo by in-vitro experiments shall preferentially use primary cells. In this study we compared non-modulated (CW) and modulated WMR exposure of biological cells in-vitro. Human ADMSC (Adipose-Derived Mesenchymal Stem Cells) were exposed to very weak non-thermal levels of microwave Electromagnetic fields at 1135 MHz ,SAR (Specific Absorption Rate) ≈ 0.002 W/kg (Watt per Kilogram) for 30 minutes daily for 4 days. A statistically significant decrease in proliferation rate of these stem cells was observed compared to the control group with no exposure. When amplitude-modulated exposure (15 Hz (Hertz) with a depth of 80%) was used with the same carrier frequency of 1135 MHz (Mega Hertz) and consistent average power, the cell numbers showed no statistically significant difference from the non-modulated exposure, but were nevertheless lower than the not-exposed control. The observed decrease in proliferation in response to -weak microwavefields supports the hypothesis that non-excitable cells, such as undifferentiated mesenchymal stem cells can interact with, and respond to weak electromagnetic radiation at *communication frequencies*. Possible mechanisms responsible for the observed results have been hypothesized and directions provided for future research.

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1. INTRODUCTION

Experimental research relating the effects of weak microwave radiation on cells have received increasing attention mainly due to health concerns in recent years. While strong microwave EM fields have been known to produce thermal effects because of the energy dissipation in tissues, it has been suggested that weak, non-thermal fields interact with cells through affecting the body's natural *information exchange system* which has been found to be partly electrical [1, 2]. An important question is whether microwave EM fields amplitude-modulated with low frequency waveforms can be demodulated by cells, especially through nonlinear mechanisms in the cell membranes. These topics have received increasing attention in the literature, with studies reporting mixed results ranging from complete positive to complete negative [3-9]. It can be supposed that the cell type under study in *in-vitro* experiments can be a crucial factor in being able to capture a real effect on cell proliferation and/or behavior. Most in-vitro samples in the litrature come from cancerous or immortalized cell lines, or from specialized cells, and relatively few works have been undertaken with primary or stem cells [10-12]. It is the authors' belief that results concerning stem cells might prove to be much more revealing for the effects of radiation on living cells. In addition to their relatively higher degree of freedom, stem cells are distributed within the body , are key regulators of regeneration and are capable of reproduction. It has recently come into light that reproduction and proliferation are affected by electric fields, both at low and high frequencies. While cancerous cells also share some of the abilities of stem cells, notably reproduction and low transmembrane voltage, they deviate in many aspects from normal healthy cell behaviors, especially those relating to growth control. So over-relying on cancerous cells

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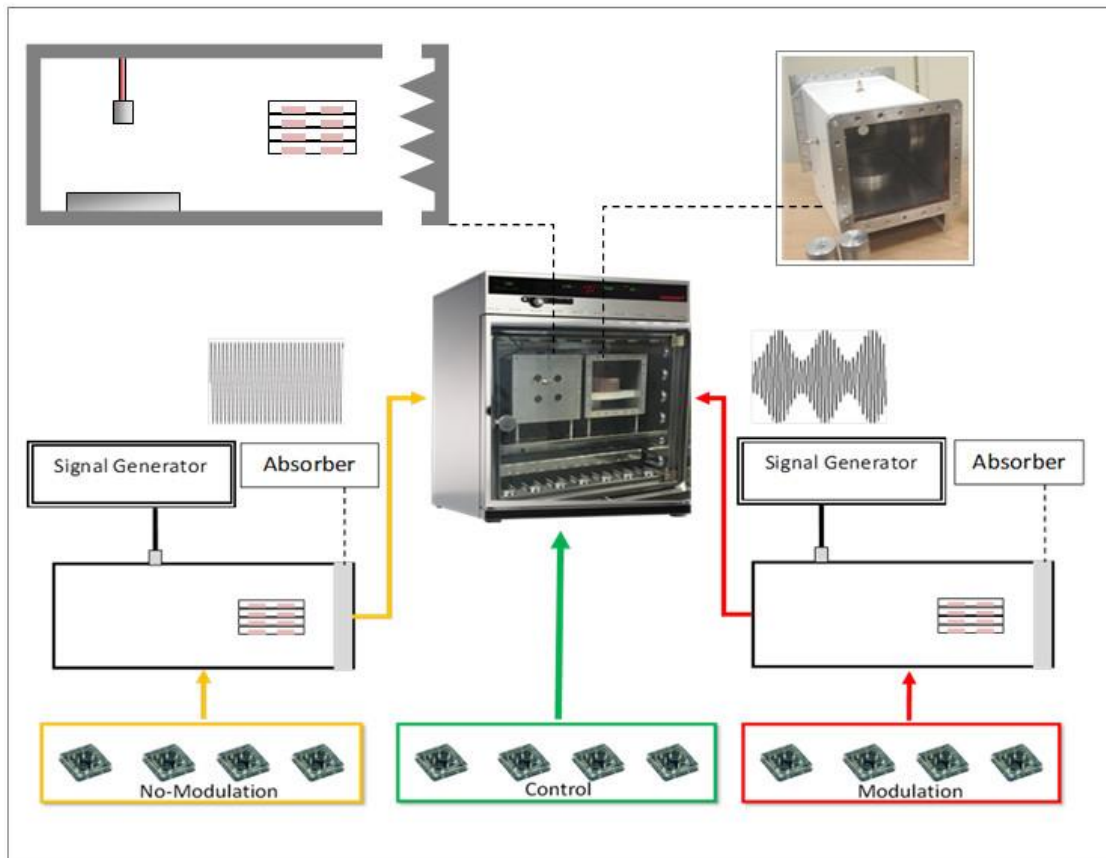

Fig 1. The applicator geometry and placement of the samples (Top). The placement of the applicators within the Incubator (Middle). graphic picture of exposure set-up where the plates located in applicator and absorber has been installed (left and right). During exposure, the aperture of the control applicator is also closed by a metallic plate to ensure similar convection and ambient temperature for exposure and control samples. The modulated and non-modulated exposure are performed consecutively and controls are stored in the inactive applicator during each exposure.

experimental results might easily become misleading.

There are very few researches carried out on stem cells exposed to microwave EM fields. However a major part of these studies have employed relatively strong exposure levels around 0.4-2 W/kg SAR [10, 12] which correspond to maximum permissible exposure according to safety regulations [13]. This paper has its novelty in studying *stem cells* exposure to *weak*, non-thermal levels of modulated and non-modulated microwave EM fields. A discussion on the possible mechanisms governing these non-thermal effects is presented through likely effects on calcium signaling pathways or changes in one of the cell cycle checkpoints. Specifically, we discussed how fields might interact with signaling pathways that control cell behavior.

2. MATERIALS AND METHODS

2.1. Culture of ADMSCs

Adipose mesenchymal stem cells (ADMSCs) were donated by a collaborated lab where the type of cells is validated by specific markers. Briefly these cells were isolated from human adipose tissue. Adipose tissue obtained from cosmetic liposuction was treated with 0.2 % collagenase II under intermittent shaking at 37 °C for 30 minute. After centrifugation, the supernatant was discarded and the cell pellet was re-suspended in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10 % (v/v) FBS (Fetal Bovine Serum) and 1% penicillin/streptomycin (Gibco). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and the medium was replaced every 2 days. When cells reached about 80% confluency, they were detached and seeded into 4-well plates at a density of 10^4 cell/well.

2.2. Exposure Setup and Characterization

2.2.1. Setup

The exposure setup consists of two in-house rectangular waveguide applicators designed for the 800-1200 MHz frequency band. The applicators were placed in a CO_2 Incubator (Memert INC108). Only one applicator was excited during exposure while the other one remained inactive as control samples. Four stacked four-well plates were placed at the cross-sectional center of the waveguide (Fig.1). The incubator was kept at 37 °C with a CO_2 level of 5% for the entire test duration of 4 days. Samples were taken out shortly on odd days for replacement of culture medium.

Each applicator is a waveguide (aperture, 33 cm long) as depicted in Fig 1 and described by the authors in detail

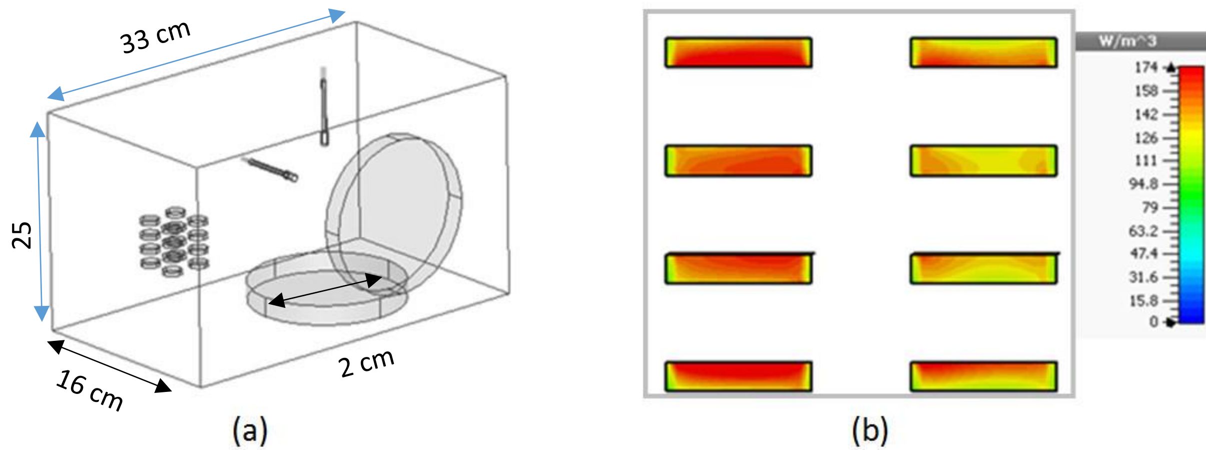

Fig 2. (a) The computational model for the experiment in CST-MWS (b) Computational results showing electric field loss densities within the volume of the culture medium assuming 1 watt input power. The Horizontal probe is excited. Our actual power is 11.2 dBm, for which the results in this figure shall be scaled down.

elsewhere [14]. The applicator can be excited by probes at one end. By providing wave-absorbers at the other end of the active applicator during exposure periods, it practically supports traveling waves, and resonator modes are mostly avoided.

The aperture of the control (inactive) applicator is also covered by an aluminum sheet during activation of the active applicator, in order to maintain similar environmental and convectional conditions for both test and control groups. The absorber and cover are removed from the applicators at non-exposure times.

We choose a sinusoidal signal of 15-Hz frequency for our amplitude modulation since the frequency range 14-16 Hz has been shown to be a selective window for non-thermal effect [15,16]. The power input to the applicator for non-modulated exposure was 11.2 dBm (DeciBels below 1 Milliwatt). To keep a consistent average power for the modulated exposure (15 Hz, Modulation depth 80%), the pre-modulation carrier power has been calculated and set to 10 dBm.

2.2.2. Dosimetry

In order to evaluate the dosimetrical characteristics of our exposure setup a Computational model of the entire applicator in the present of plates was created using the commercial electromagnetic simulator CST-MWS (Computer Simulation Technology-Microwave Studio) (Fig 2). The software solves for the electric fields everywhere within the applicator, including the culture plates. The electrical properties of the culture material are assigned as real relative permittivity of 75, with a total conductivity (dielectric losses and ionic contributions) of 1.2 S/m (Siemens per Meter). The inner walls of the applicator are simulated as perfect electrical conductors (PEC).

For the 11.2 dBm input power to the applicators, the mean loss density within the culture medium is obtained as being between 1.4 and 2.3 W/m^3, corresponding to a SAR of 1.8 mW/Kg and a mean E-field strength of 1.2 V/m , which are the assumed values experienced by the cells.

2.2.3. Timing and SAR Level

Exposure was applied continuously for 30 minutes daily for 4 consecutive days. One sample is taken at the end of every observation day for cell counting and proliferation assay. An Upper bound on SAR within the exposure group is estimated computationally (Fig 2) to be 2.27×10^{-3} W/Kg through detailed simulation. This SAR results in an upper bound of temperature rise as 1×10^{-3}°C for 30 minutes exposure time as found using:

$$\frac{SAR}{c}=\frac{\Delta T}{\Delta t} \qquad (1)$$

This is a relatively insignificant temperature rise, which is why the observed results can be considered as being *non-thermal* in nature.

2.3. Cell proliferation assay

The effects of WMR frequencies exposure on cell proliferation were evaluated by MTT assay. cells were cultured in 4 well plates with a density of 10,000 cells per plate one day before exposure to WMR. The active and exponentially growing cells (though not necessarily synchronous or in-phase) were exposed to WMR according to the procedure discussed in 2.2.3. 24 hours after the last exposure, the medium of 3 exposed wells were discarded, cells washed twice with PBS and replaced with 270 µl of serum free medium and 30 µl MTT solutions (5 mg/ ml in PBS). Then, they were incubated for 3 hours at 37°C in a 5 % CO_2 incubator. The medium was removed and 300 µl of DMSO (Sigma-Aldrich) was added to each well and mixed thoroughly using a pipette and incubated in a dark room for 20 minutes. Afterwards, 100 µl of medium was transferred to a 96 well plate and absorbance of each well was read at 570 and 630 nm wavelengths as test and reference wavelengths, respectively, by ELISA reader. The results were presented as OD (Optical Density). Cell viability is represented through difference of absorbance at the two wavelengths. The number of viable cells is directly

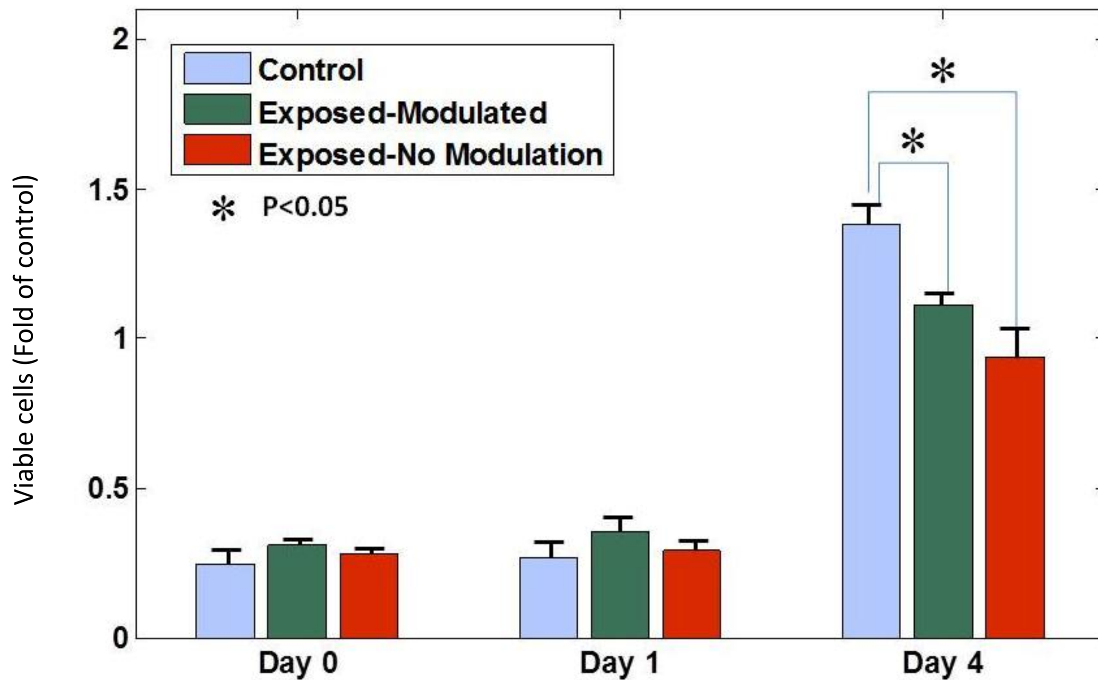

Fig 3. Results of cell-proliferation assay expressed as *exposed samples relative to control* optical density (OD). The experiments were repeated at least three times. * represents P < 0.05 in comparison with the control group

Table 1. summary of the Anova analysis of cell-proliferation on the fourth day.

Final Day	Control	Modulation	No-Modulation	Anova
Mean	1.38	1.109	0.939*	F_s=20.978
St.Dev.	0.07	0.15*	0.06	P=0.0027

Result were evaluated by MTT assay and represented as *exposed samples relative to control* optical density (OD). * represents P < 0.05 in comparison with the control group

proportional to the total amount of formazan produced. In parallel, a group of cells were sham-exposed as the negative control. The experiment was repeated at least three times, but only the last and most reliable round of experiments are reported here.

3. RESULTS

Consistent decrease in proliferation was observed in exposed samples relative to control (Fig 3). Results showed that on the fourth day, the mean difference between control and exposed groups either Modulated or Non-modulated cases was statistically significant ($P < 0.05$). Table 1 has provided a summary of the Anova analysis (Welch's multi-group Anova). This analysis supports statistical significance of radiation effects on the fourth day (P=0.003).

4. DISCUSSION

In our study we found that WMR decreased cell proliferations. This observation is similar to what Shahbazi-Gahrouei and coworkers reported. They exposed human adipose-derived stem cells to GSM cellular phones 900 MHz frequency with intensity of 354.6 µW/cm2 [12]. However, they did not study non-modulated waves and used a higher power density than ours. In parallel Jooyan and coworkers did not find any changes in chinese hamster ovary (CHO) cells viability after 12 or 24 hours exposure to 900 MHz frequency (CW RF fields) [17]. Also umbilical cord blood (UCB) cells did not show significant difference in apoptosis between sham-exposed and samples exposed to microwave pulsed signals from GSM900/UMTS test-mobile phone with output power of 0.25 W for 3,1-3 or17 hours [18]. These seemingly contradicting results might confirm that the difference between the cell lines is a main factor in theimpact of exposure. Nevertheless, the importance of exposure protocols should not be ignored.

Considering the significance results, now the question is : what is the mechanism of the significant decrease of proliferation in response to an ultra-weak microwave exposure? Since microwave fields have low-energy photons, direct chemical effect is unlikely. A cell is, however, a structure far from thermodynamic equilibrium, i.e. with internal storages of energy. This implies that for EMF to have an effect, it only needs to act as a trigger, though with energy levels far below that of the actual effect.

There is converging evidence that the effects of both low-frequency and high-frequency, microwave Electromagnetic fields can be through Ca^{2+} signaling pathways, via activation of VGCCs (voltage-gated calcium channels) in the membrane. This seems rational since the membrane is a well-known amplification point for the applied electric fields [19] and VGCCs naturally respond to this voltage. It is also known that increased intracellular calcium levels have profound roles in proliferation, differentiation, and apoptosis. Several studies have shown that certain EMF effects can be blocked or lowered by introducing chemical VGCC blockers, among them a study of Ca^{2+} Dynamics in Stem-cell derived neuronal cells at 700-1100 MHz [20,21]. It has also been shown that intracellular levels of Ca^{2+} and NO (Nitric Oxide) concentrations increase during therapeutic bone-growth stimulation [19]. Considering the VGCC model, following the activation of VGCC and increased intracellular Ca^{2+} multiple regulatory mechanisms can follow, but among them, there is a pathway with negative consequences (cell-death). This pathway, which can be considered pathophysiological, happens when NO forms peroxynitrite after reacting with superoxide. Peroxynitrite is a potent oxidant, which can produce radical products such as NO_2, which can possibly lead to DNA damage. The VGCC model can thus accommodate both therapeutic and pathological effects, depending on the cell-type, growth stage, etc [19]. A plausible justification for the observed non-thermal effects can be based on this pathway, where cells might experience oxidative stress from the perturbed calcium concentration with consequent cell-death as a result. Additionally, another hypothesis is also worth mentioning in[18]. A more detailed study is needed to substantiate and clarify the related pathways for the observed effects. Investigation of changes in intracellular calcium level and cell viability in different cell cycle phases with synchronization during WMR exposure are our goals for future studies.

5. CONCLUSION

ADMSCs showed statistically significant decrease in proliferation in response to exposure to an AM (Amplitude modulated) modulated and non-modulated sinusoidal time-varying electromagnetic field with a frequency of 1135 MHz (30 minutes daily for 4 days) at a non-thermal level of SAR~0.002 W/kg. The observed results support decreased proliferation in-vitro. Calcium-related processeshave been suggested as a potential mechanism for the observed effects.

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NOMENCLATURE

μ Micro

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