ORIGINAL ARTICLE

Electromagnetic Field Effect on Bone Marrow Stem Cells Differentiation and Nucleoli AgNOR

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Received: 14 March 2022/ Accepted: 28 April 2022

Abstract

Purpose: Numerous studies have described the effect of Electromagnetic Fields (EMFs) in the promotion of Bone Marrow Stem Cell (BMSC) differentiation. We aimed to investigate the influence of frequency (10 and 100 Hz) and different pulse shapes (sine, rectangular, and triangular) of EMF on rats' BMSCs.

Materials and Methods: The BMSCs in 6 groups were exposed to EMF for 1 h/ 7 days. The BMSCs viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. The cresyl violet labeled the Nissl bodies, and the silver nitrate staining was done to evaluate the BMSCs nucleoli AgNORs.

Results: The MTT test verified that EMF and pulse shape did not affect cell viability. In Nissl bodies staining most of the large neurons were related to the rectangular 10 Hz EMF group. The majority of the differentiated BMSCs were astrocytes, microglia, and oligodendrocyte in the triangular 100 Hz EMF group. Although the silver nitrate staining confirmed the effect of 10 Hz EMF, pulse shape alteration did not affect AgNOR parameters. In conclusion, we presented a low-magnetic flux density EMF (400 μT) to assess the responses of BMSCs nuclei.

Conclusion: The findings showed that BMSCs differentiation was frequency-dependent. Further investigations are recommended for recognizing the function of EMF on BMSCs.

Keywords: Electromagnetic Field; Bone Marrow Stem Cell; Cell Viability; Differentiation; Ag Nucleolar Organizer Regions.

DOI: https://doi.org/10.18502/fbt.v10i2.12217

Frontiers in BIOMEDICAL TECHNOLOGIES

1. Introduction

The magnetic field can be time-independent (static) or time-varying (electromagnetic). The effect of Electromagnetic Fields (EMFs) has been described as a tool to promote the proliferation and differentiation of Bone Marrow Stem Cells (BMSCs) [1]. Results are displayed at low frequencies (30-300 kHz) and extremely low frequencies (3-30 Hz) [2]. EMFs appear to have significant effects on cells, tissues, and biological processes such as regeneration, wound healing, and cell migration; furthermore, proliferation and differentiation of BMSCs into neurons are essential for the healing of neurodegenerative disorders [2].

Several studies have shown that EMF at 50 Hz induces a decrease in cell proliferation [3]. Other researchers have reported increased cell proliferation at 10 Hz and 15 Hz EMF [4-6]. In addition, 50 Hz EMF exposure promotes the cell cycle to S-phase, and Deoxyribonucleic Acid (DNA) replication [7] and EMF (100 Hz) have been shown to cause cell cycle blockage [8]. Other reports conclude that EMF radiation (25 and 60 Hz) does not affect cell cycle changes or apoptosis [9, 10].

The cell nucleoli consisted of Nucleolar Organizer Regions (NORs) that are active portions of chromosomes. These proteins are involved in the ribosomal synthesis and are argyrophilic (AgNOR) during the interphase, hence can be easily visualized as black dots after colloidal silver staining [11]. The amounts of silver-stained dots identify the amount of NORs associated with protein synthesis, thus demonstrating the proliferative capacity of a cell [12]. The AgNOR parameter (the number, mean nucleus area, shape, and size of AgNOR dots) has been suggested for evaluating the cell proliferation rate [13]. The Okudan study has shown that ≤5 µT magnetic flux densities did not change AgNOR numbers in the EMF exposed cells [14].

EMF has some parameters (frequency, field strength, time, and also the magnetic field pulse shape like sine, rectangular, and triangular) that can affect the results. Previous studies have specified neural cell differentiation in EMF exposed (50, 60, 75, and 100 Hz) BMSCs. The findings admitted differentiation to neurons in sine pulse shape EMF [15-17]. While, for astrocytes, the increase of BMSC differentiation was observed in the low flux density EMF (400 µT) with a rectangular pulse shape form (75 Hz) [6].

Most stimulating EMFs have sine waveforms because of 50 Hz or 60 Hz alternating currents, but, there is a lack of findings on the effectiveness of various pulse shapes of 10 and 100 Hz EMF on BMSCs. Therefore, the purpose of this study was to investigate the effect of EMF frequency and pulse shape on the rat BMSC viability and morphology. Accordingly, the novelty of this research is the control of the low flux density EMF (400 µT) and frequency while investigating the effect of the pulse shape (sine, rectangular, and triangular) on the viability and morphology of BMSCs to determine the EMF window effect.

2. Materials and Methods

2.1. BMSCs Culture

BMSCs were extracted from the hind limbs of four healthy adult male Wistar rats (12-13 weeks of age) in a sterile condition. Bone marrow cells were seeded onto Phosphate Buffered Saline (PBS) (1038500, Gibco, USA), followed by incubation in Dulbecco's Modified Eagle Medium (DMEM) (1/043 g L-Glutamine, 11530556, Gibco, USA), 20% FBS (10270106, Gibco, USA), and penicillin-streptomycin (Bioidea, Iran) in 25-cm^2 flasks at 37°C and CO_2 5%. Purification of BMSCs was managed for three passages. The viability and cell density of harvested BMSCs were assessed using a trypan blue exclusion method and 5×10^4 cells in a 3.5 cm plate incubated to EMF exposure.

2.2. Electromagnetic Field Apparatus and Exposure

A signal generator (GFG-8019G, Goodwill, Taiwan) supplied frequency and current pulse shape (sine, rectangular or triangular) that was checked by a digital oscilloscope (Topward 9022, Taiwan). The electrical current is amplified by a 35 W acoustic amplifier (ALFA, Navasaz, Iran), and the regulated current is assigned to a pair of coils (300 turns, internal diameter of 16 cm) which are fastened by rectangular Plexiglas plates (20 × 20 cm, 6 cm distance). Figure 1 shows the experimental EMF exposure set-up. Uniformity of the magnetic field was performed using the EMF meter EMF-827 (LUTRON Electronic Enterprize Co. Taiwan) with a Hall Effect sensor (Accuracy ± 4% for 3-300 Hz). The distribution of magnetic flux density in the exposure system was shown in Figure 2. Magnetic flux density in the center

of the Helmholtz coil (Mean 400 ± 4 µT) was regularly checked before each BMSC exposure. The Helmholtz coil was placed in the middle of a standard CO_2 incubator (5% CO_2, 37°C). One harvested BMSCs flask was placed at the center of the Helmholtz coil which has a homogenous magnetic field. The experimental BMSCs in 6 groups were exposed to EMF (400 µT, 10 and 100 Hz) with sine, rectangular or triangular pulse shape for 1h/7 days. The performance of the Helmholtz coil during the cell exposure did not affect the incubator temperature. The situation of the sham group was the same as other groups, except that the current of the coils was turned off.

Figure 1. Experimental Electromagnetic Field (EMF) exposure set-up

2.3. Staining Methods

The estimation of BMSCs viability was carried out by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (M5655, Sigma, USA). Briefly, experimental BMSCs were incubated in the MTT solution (5 mg/ml, 3 h at 37ºC) and data was interpreted in 570 nm wavelength by an Eliza reader (A.D. TOUCH, apDia, China). To assess the morphology of EMF exposed BMSCs, the cresyl violet staining labeled the Nissl bodies. Briefly, BMSC hydrated slides were immersed in a cresyl violet stain (0.1%-Sigma, USA) for 3 min. The Nissl bodies appeared as dark-violet spots around the cells' nuclei. The silver nitrate staining was done to evaluate the BMSCs nucleoli AgNORs. The working solution was made up of two parts of 50% silver nitrate and one-part mixture of 2% gelatin and 1% formic acid solution (prepared with distilled water immediately before the staining procedure) and incubated at 37°C for 15 min [11].

2.4. Statistical Analysis

Statistical analysis was accomplished by computer using SPSS16.0 software. One-way ANOVA and Tukey post hoc multiple comparison tests were used to analyze the MTT and AgNORs data. A *P*-Value less than 0.05 ($P < 0.05$) was considered to be statistically significant.

3. Results

To assess the EMF effect on BMSCs viability, MTT dye was utilized. According to Figure 3, the findings of the MTT assay appear that EMF exposure in frequencies of 10 and 100 Hz had no significant result on cell viability in experimental groups versus the sham group ($P > 0.05$). Furthermore, to qualify the BMSCs undergoing in vitro trans-differentiation, samples were stained with cresyl violet in all trial groups (Figure 4). The findings of BMSCs samples showed some non-differentiated cells as the primary neuron, microglia, and oligodendrocyte. In the 10 Hz EMF exposed groups, large neurons were seen

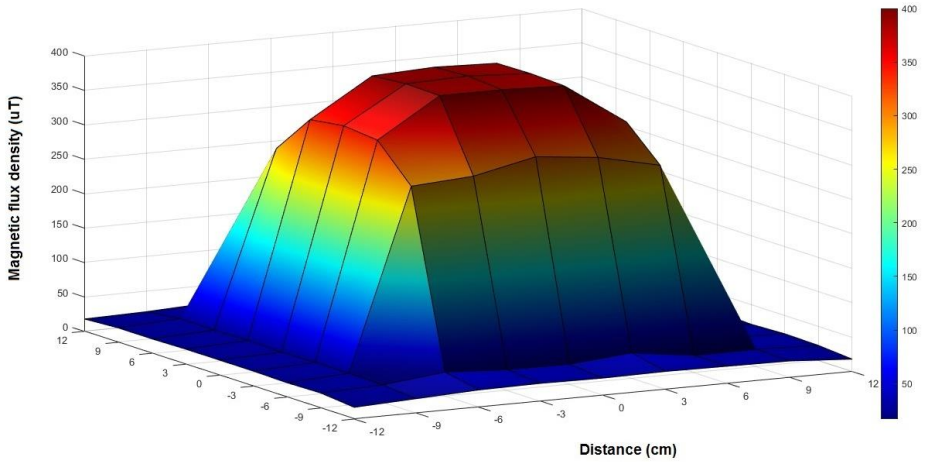

Figure 2. Magnetic flux density distribution in the EMF exposure system

with apparent synapses. The most of large neurons were related to rectangular 10 Hz compared with sine and triangular 10 Hz EMF groups. Even though a few small neurons were seen within the 100 Hz EMF groups, most of the differentiated cells were astrocytes, microglia, and oligodendrocyte. Furthermore, in the 100 Hz EMF experimental groups, the majority of the large neurons were related to triangular pulse shape compared with sine and rectangular pulse shape groups.

The rectangular EMF waveform caused neural differentiation but the sine and triangular pulse shape EMF induced glial differentiation. Briefly, the findings of cell morphology showed the EMF ability in BMSC differentiation to neural cells.

The BMSCs nucleoli AgNORs were evaluated by the silver nitrate staining. As shown in Figure 5, the AgNORs dots appeared as brownish intra-nuclear spots on a pale yellow background. The AgNOR parameters of 100 nuclei in 5 individual areas of samples were recorded. The nuclear area (μm^2), total AgNOR area (μm^2), the total number of AgNOR dots, and the ratio of total AgNOR area to the nuclear area were calculated as the AgNOR parameters. In the study of AgNOR parameters, we observed a significantly decreased nuclear area in

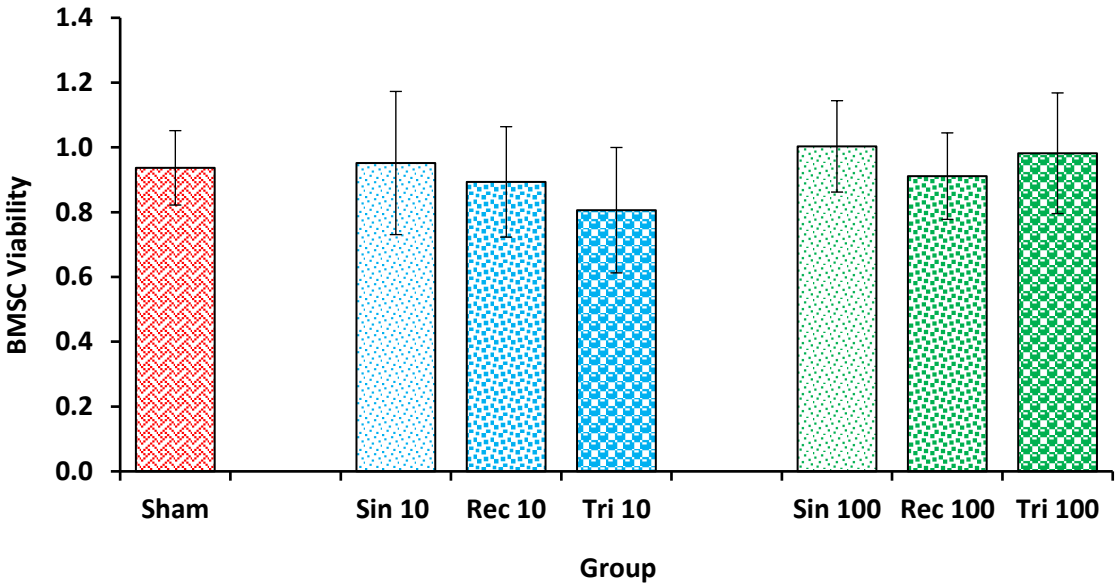

Figure 3. The Bone Marrow Stem Cells (BMSCs) viability which was evaluated by the MTT test. (Sin) Sine, (Rec) Rectangular, and (Tri) Triangular pulse shape experimental groups. The findings verified that EMF (10 and 100 Hz), and pulse shape did not affect cell viability. The results represent the mean ± SD as a bar chart

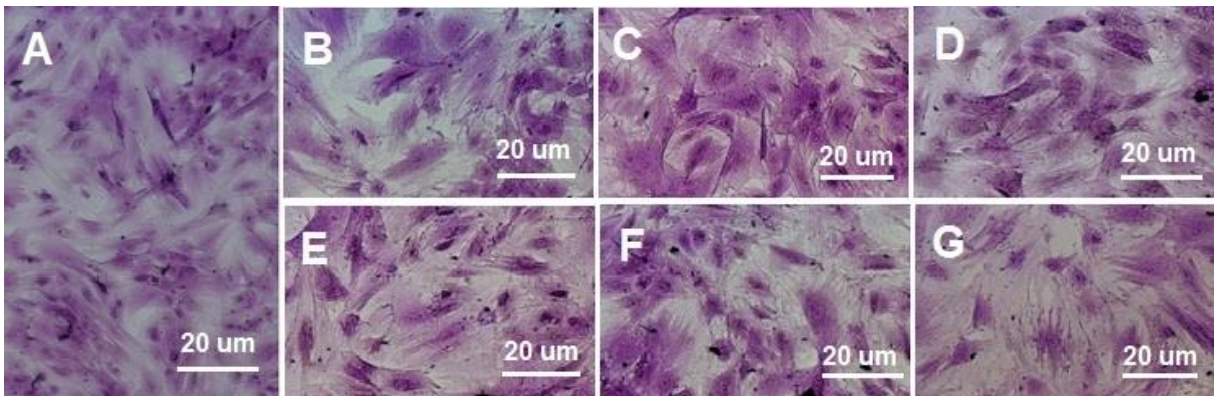

Figure 4. Morphology of BMSCs after cresyl violet staining of experimental groups: (A) Sham, (B) Sine 10 Hz, (C) Rectangular 10 Hz, (D) Triangular 10 Hz, (E) Sine 100 Hz, (F) Rectangular 100 Hz, and (G) Triangular 100 Hz. The data confirmed maximum cell differentiation to large neurons in the rectangular 10 Hz EMF group, while the majority of differentiated cells were astrocytes, microglia, and oligodendrocyte in the triangular 100 Hz EMF group. (x 400)

10 Hz EMF exposed groups compared to the sham group ($P < 0.005$). The 100 Hz EMF group's nuclear area was the same as the sham group (Figure 6a). In response to the EMF effect ($P < 0.005$), no difference was observed in the pulse shape in the nuclear area of the same exposed groups. In the data analysis, the total AgNOR area was significantly reduced in the 10 Hz groups compared to the other groups ($P < 0.05$). However, the total AgNOR area of the 100 Hz groups that responded to the same field intensities had no changes and was similar to the sham group (Figure 6b). Change of pulse shape has no significant difference between total AgNOR area in the same EMF exposed groups ($P > 0.005$). As shown in Figure 6c, the EMFs affect the total AgNOR numbers of the experimental groups. The results confirmed the maximum total number of AgNOR dots in 10 Hz exposed groups with a significant difference compared with other experimental groups ($P < 0.05$). Meanwhile, the total number of AgNOR dots decreased in 100 Hz groups compared to the sham group ($P < 0.05$). As illustrated in Figure 6d, the proportion of total AgNOR area to the nuclear area of 10 Hz groups was significantly lower than those of the other ones. The result of 100 Hz EMFs was the same as the sham group. Overall, AgNOR staining confirmed the effect of 10 Hz EMF on BMSC proliferation rate and so pulse shape alteration did not affect AgNOR parameters.

4. Discussion

EMF exposure can persuade the capacity for self-renewal and neurogenic potential of BMSCs [2]. In this study, we proposed to evaluate the influence of different frequencies (10 and 100 Hz) and pulse shape alteration (sine, rectangular, and triangular) of EMF on rat BMSCs viability and morphology.

MTT staining results show that exposure to EMF (10 and 100 Hz, 400 µT) and pulse shape variation (sine, rectangular, and triangular) do not affect BMSC viability. The results of the Ross *et al.*'s study have shown that EMF (5 Hz, 400 µT) does not affect cell viability and proliferation rate of a human mesenchymal stromal cell line [18]. But Asadyan *et al.* and Mahna *et al.*'s results confirmed a decrease in cell viability in groups exposed to EMF (50 and 60 Hz) [6, 19]. Other investigators reported decreased cell viability caused by EMF-induced cell apoptosis and necrosis [5, 20].

The morphology assessment of BMSC samples showed some non-differentiated cells as the primary neuron, microglia, and oligodendrocyte. In the 10 Hz EMF exposed groups large neurons were seen with apparent synapses, and so BMSC differentiation to small neurons, astrocytes, and oligodendrocytes aroused with the increase of frequency (100 Hz). The rectangular pulse shape caused neural differentiation but the sine and triangular pulse shape EMF induced glial differentiation. In harmony, the findings of Urnukhsaikhan *et al.* (60 Hz), Bai *et al.* (50 Hz), and Ho *et al.* (Static EMF) admitted differentiation to neurons [17, 21, 22]. While increment of BMSC differentiation to astrocytes was seen in Asadian *et al.*'s (75 Hz) study [6].

In this study, AgNOR parameters confirmed the effect of 10 Hz EMF on BMSCs, while the 100 Hz EMF results were the same as non-exposed sham cells. Moreover, pulse shape alteration did not have any apparent effect on EMF exposed cells. NORs are a makeup of ribosomal DNA and proteins. The intracellular AgNOR parameters provide information both on cell morphology and cell

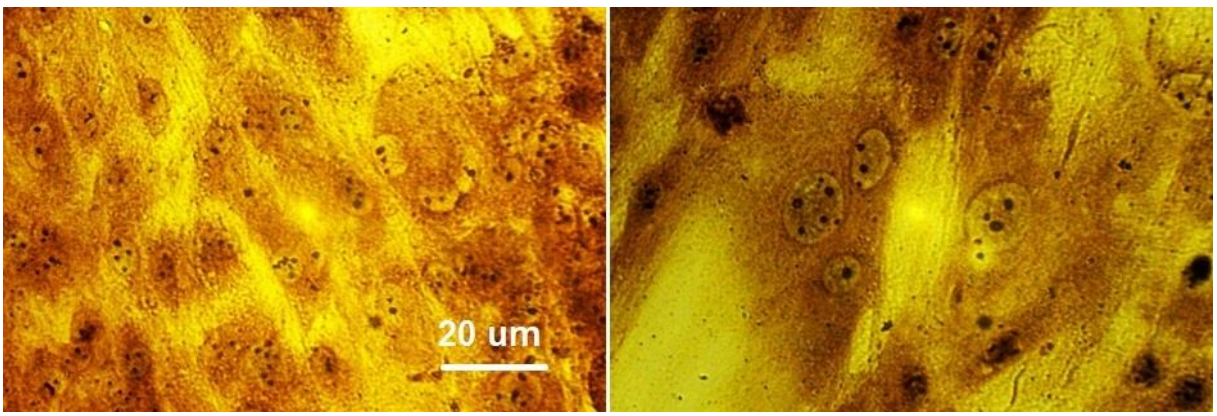

Figure 5. Ag Nucleolar Organizer Regions (AgNORs) dots (brownish intra-nuclear spots) in BMSCs. (x 400)

behavior. Hence, the AgNOR evaluation can present an index of cell proliferation. In agreement, researchers such as Liu *et al.* (10 Hz), Sun *et al.* (15 Hz), Bai *et al.* (50 Hz), and Tu *et al.* (15 Hz) have registered BMSC proliferation increment [1, 4, 5, 21].

Stem cells' response to the EMF stimulation is dependent on their situation of differentiation and it may be that EMF moderates the action of transcription factors and the level of cell cycle regulatory genes [4]. It suggested that EMF could cause a cell cycle blockage, and impairs the synthetic mechanisms at 100 Hz [8]. But after EMF radiation 25 and 60 Hz, neither cell cycle alteration nor cell death was seen in fibroblasts and cancer cells [9, 10]. Moreover, Focke *et al.*'s data confirmed the EMF 50 Hz effect on DNA fragmentation [7]. Accordingly, EMF radiation (1 Hz, and 60 Hz) induced activation of p38, cell death pathways, and an increase in cell line apoptosis [23, 24]. However, the results of other researchers showed that EMF (60 Hz) could not elicit DNA damage in the

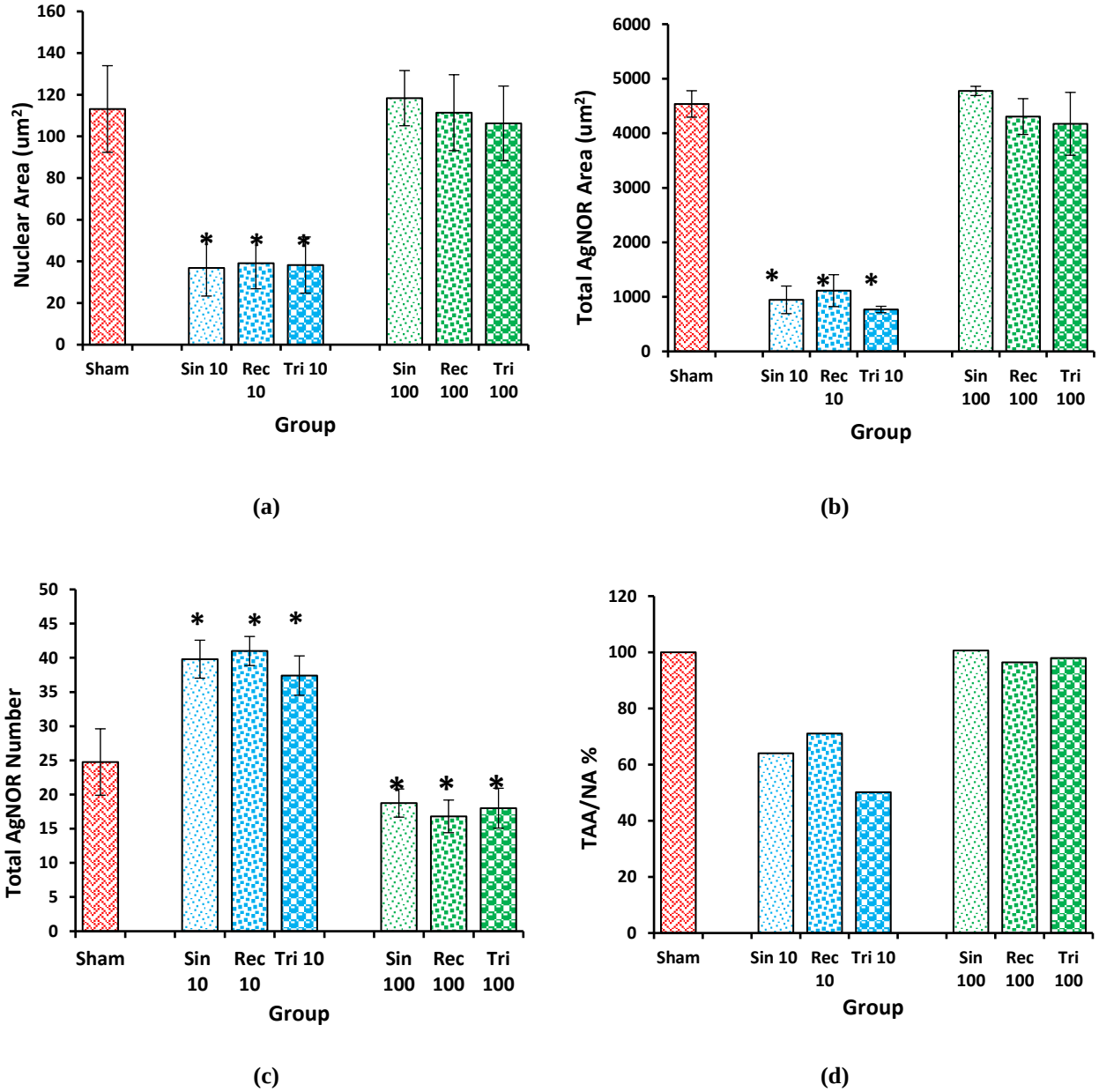

Figure 6. The BMSCs nucleoli AgNOR parameters: (a) The nuclear area (µm²), (b) the total AgNOR area (µm²), (c) the total number of AgNOR dots, and (d) the total AgNOR area/the nuclear area. Overall results confirmed the effect of 10 Hz EMF on BMSCs nucleoli AgNOR parameters. The results represent the mean ± SD as a bar chart

* A significant difference compared to the sham group ($P < 0.05$)

mice's brain and human blood cells [25, 26]. In the Akdag *et al.*'s study, long-term exposure EMF could provoke ROS creation but did not affect cell apoptosis [27]. In addition, uniform EMF exposures caused mild intracellular Reactive oxygen species (ROS) levels that were followed by an increment of cell proliferation [2, 10]. However, Mattsson *et al.* concluded low concentrations of ROS which can activate signaling cascades and subsequently gene expression, and cell proliferation [28]. However, the mechanism of the EMF stimuli (the frequency, magnetic flux density, pulse shape, and EMF duration) to promote an effective consequence on proliferation would be assigned for each cell type. With adequate temporal components, it could have a positive effect on cell proliferation and differentiation. In the present study, the effect of 10 Hz, 400 µT EMF on BMSC viability was reported. The EMF responses were frequency-dependent, and pulse shape alteration did not affect AgNOR parameters. Meanwhile, in samples stained with cresyl violet, the rectangular EMF caused neural differentiation, and the sine and triangular pulse shape EMF induced glial differentiation. These results may be confirming the EMF "window effect" on BMSCs. However, there are still uncertainties about the mechanisms of the EMF, and wish further investigation could recognize the function of EMF on BMSCs proliferation and differentiation.

5. Conclusion

In conclusion, we presented a low flux density EMF (400 µT, 10, and 100 Hz) to assess the frequency-dependence of BMSCs responses. The findings indicated that BMSCs differentiation was frequency-dependent, and the largest neurons were induced in rectangular 10 Hz EMF. Although in the 100 Hz EMF exposed groups some small neurons were seen, most of the differentiated cells were astrocytes, microglia, and oligodendrocyte.

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