

## Original Article

# Effects of 50 Hz electromagnetic fields on mitochondrial DNA copy number of mesenchymal stem cells with different states

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**Abstract:** Mitochondria play pivotal roles in a range of cellular functions. When mitochondria underwent significant changes during cellular differentiation, mitochondrial DNA (mtDNA) copy number was elevated. Many reports showed that ElectroMagnetic Fields (EMF) could affect Mesenchymal Stem Cells (MSCs) differentiation. However, the effects of EMF on mitochondrial DNA (mtDNA) copy number of MSCs have not been discovered. In this study, rat MSCs were isolated from bone marrow and divided to culture with three kinds of conditioned media, then exposed to EMF. EMF groups were exposed to 50 Hz EMF at 5 mT of flux densities for 30 min, 1 h or 6 h for 2 days. Control cultures were performed without EMF stimulation. The effects of EMF on mtDNA copy number were investigated. The results revealed that a transitory decrease in mtDNA content was observed during early osteoblastic differentiation of MSCs, but a sharp increase after neuronal induction for 3 days. After EMF exposure for 2 days, we found that 50 Hz EMF increased mtDNA copy number of MSCs in the pluripotent state and in a time-dependent manner during osteoblastic differentiation. During neuronal differentiation, mtDNA replication was enhanced after EMF exposure for 30 min, and then a gradual decrease in mtDNA content was observed after EMF exposure for 1 h and 6 h, respectively. Those results indicated that 50 Hz EMF had different effects on mtDNA copy number of MSCs with different states.

**Keywords:** Mesenchymal stem cells, electromagnetic fields, mitochondria DNA copy number, cell differentiation

## Introduction

Mitochondria are intracellular organelles that consistently fuse with each other to form a tubular shape and divide into smaller fragments, and generate adenosine triphosphate and reactive oxygen species (ROS). Recently, some reports suggested that mitochondria possessed important roles on stem cells [1, 2]. As one of the common biomarkers, mitochondria DNA (mtDNA) is susceptible to intracellular condition and is associated with cell fates and development. Previous evidences suggested that mtDNA content was elevated when mitochondria underwent significant changes during cellular differentiation [3, 4]. A low amount of mtDNA copies were detected in undifferentiat-

ed embryonic stem cells, while this number increased upon differentiation [5].

Many researchers demonstrated that electromagnetic fields (EMF) could alter cellular processes by increasing intracellular reactive oxygen species (ROS) concentrations [6, 7] and affected many types of cells proliferation and/or differentiation [6, 8, 9]. Mesenchymal stem cells (MSCs) is a promising candidate cell type for regenerative medicine, and EMF can affect MSCs proliferation or differentiation at different states [10, 11]. Furthermore, ROS production increase during differentiation of human MSCs into neurons had been found by Jeong-Eun Park et al. [12]. As we know, mitochondria are the main source of intracellular ROS [13, 14], and

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ROS have close relationships with cell fate determination. Although high levels of ROS have deleterious effects on cells such as lipid peroxidation and DNA damage, low levels of ROS have positive effects on cells [15, 16]. Thus, basing on the above mentioned evidences, we hypothesized that mitochondria were the essential sites and played important roles in biochemical regulation of MSCs under EMF exposure.

The experiments reported here were designed to investigate whether 50 Hz EMF affected mtDNA copy number of MSCs or increased mtDNA copy number of MSCs during differentiation, and if so, to determine time-dependent data for this effects. The MSCs were isolated and exposed to EMF at 50 Hz for 30 min, 1 h or 6 h, respectively. Then mtDNA copy number were analyzed.

### Materials and methods

#### *Cell culture*

MSCs were taken and cultured as previously described [17]. Briefly, adherent cells derived from marrow stroma collected from the femur and tibiae of ten 8-week-old Sprague-Dawley rats (male and female, 80~120 g) were maintained as monolayer cultures and expanded using Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS, Invitrogen), penicillin (100 U/ml; Sigma-Aldrich, St. Louis, MO), streptomycin (100 mg/ml; Sigma-Aldrich), and amphotericin B (0.25 mg/ml; Sigma-Aldrich). Non adherent cells were removed by changing the medium after 24 h. The culture medium was changed twice a week thereafter. For subculture, when cells grew to 80~90% confluence, cells were detached with 0.25% trypsin/0.02% EDTA (Gibco, Grand Island, NY) and passaged at a ratio of 1:3 plates. Cells from the 3rd to the 7th passage were used for experimental purpose.

#### *Neuronal induction*

The neuronal induction protocols followed the reported paper [18]. To initiate differentiation, MSCs were seeded at a density of  $5.0 \times 10^3$  cells/cm<sup>2</sup>. When cells grew to 50~60% confluence, the cells were cultured with neuronal differentiation medium composed of DMEM/F12 with KCl (5 mM), valproic acid (2 M), forskolin

(10  $\mu$ M), hydrocortisone (1  $\mu$ M), and insulin (5 g/ml). After treatment with the differentiation medium for 1 day, we moved the culture dishes to EMF exposure system for 30 min, 1 h or 6 h, respectively. Once a day for two days.

#### *Osteoblastic induction*

According to published osteoblastic induction protocols [19], MSCs were seeded at a density of  $5.0 \times 10^3$  cells/cm<sup>2</sup>. At 50~60% confluence, the medium was replaced with OM containing (OriCell, Cyagen Bioscience, Guangzhou). After treatment of differentiation medium for 1 day, we moved the culture dishes to EMF exposure system for 30 min, 1 h or 6 h, respectively. Once a day for two days.

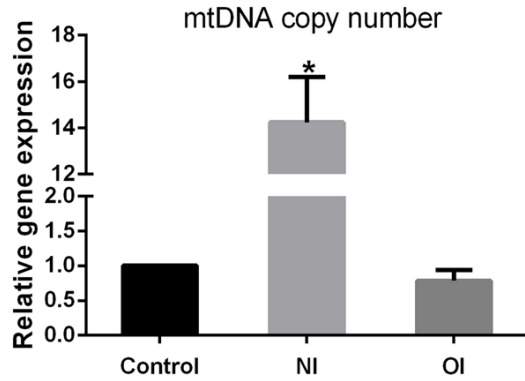
#### *EMF exposure system*

During EMF treatment (frequency 50 Hz, intensity 5 mT) [12], the magnetic intensity at the centre of coils was measured with a gaussmeter-probe (TinDun Industry Co., Ltd, Shanghai, China). The wave shape and frequency were visualized by an oscilloscope (RIGOL, Beijing, China). MSCs cultured with the various medium were divided into EMF groups and the control groups. EMF groups were given sinusoidal EMF intervention. All EMF groups were exposed to dual coils at 10 cm distance of EMF. The control groups were kept under the same conditions without EMF exposure.

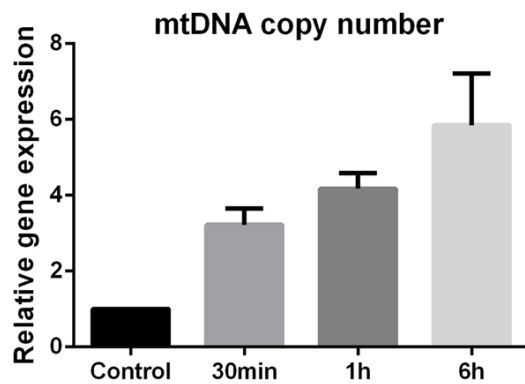
#### *QPCR*

mtDNA were isolated from MSCs using the GMS20023 kit (GENMED, Arlington, MA). Quantitative PCR was performed using kit following the manual protocol. The mitochondrial gene forward primers were 5'-CCCAACACAGG-CGTGCTT-3', and the reverse primers were 5'-ACCGCGCCGTTTAACTT-3'. The mtDNA content was corrected by simultaneously measuring rat GADD45 genomic DNA. Its forward primers were 5'-ACCCCGATAACGTGGTACTG-3' and the reverse primers were 5'-TGACCCGCA-GGATGTTGATG-3'. The thermal cycling conditions for real-time PCR were 50°C for 2 min, then 95°C for 10 min, and 40 cycles of denature (95°C, 15 sec) and annealing/extension (60°C, 60 sec). After PCR cycles, dissociation curve examination was performed. The amount of mtDNA was expressed relative to the quantity of GADD45 ( $2^{-\Delta\text{CT}}$ ) and in terms of  $\Delta\text{CT}$  values calculated.

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**Figure 1.** mtDNA copy number changed during MSCs differentiation. Total DNA was extracted from MSCs after osteoblastic induction and the mtDNA content was determined by QPCR and expressed as a fold-change from the level in the control group. The experiment was performed in triplicate. Data are mean  $\pm$  SD. NI: neuronal differentiation; OI: osteoblastic differentiation.



**Figure 2.** Effects of 50 Hz EMF on mtDNA content of MSCs for 30 min, 1 h or 6 h. Total DNA was extracted and the mtDNA content was determined by QPCR and expressed as a fold-change from the level in the control group. The experiment was performed in triplicate. Data were mean  $\pm$  SD.

### Statistical analysis

Data were represented as mean  $\pm$  SD. Multiple comparisons were performed by one-way analysis of variance followed by post Hoc corrections with Tukey's method using 11.5 SPSS statistical software. Significance was accepted with  $P < 0.05$ .

### Results

#### mtDNA copy number changed during MSCs differentiation

During MSCs differentiation, a specific remodeling of mitochondria occurred in order to meet

the energetic and anabolic demands, and the regulation of mtDNA copy number was linked with the remodeling. So we performed quantitative PCR assay on mtDNA copy number during two specific differentiation processes of MSCs. After EMF exposure for 30 min for 2 days, the results showed that a transitory decrease in mtDNA content was observed after osteoblastic induction for 3 days, but a higher increase after neuronal induction for 3 days (**Figure 1**).

#### Effects of EMF on mtDNA copy number of MSCs

To determine whether 50 Hz EMF had effects on mitochondria of MSCs with the pluripotent state, mtDNA copy number determined by quantitative PCR after 50 Hz EMF treatment for 30 min, 1 h or 6 h. The results showed that 50 Hz EMF could increase mtDNA copy number after EMF exposure for 30 min, 1 hour and in a time-dependent manner compared to the control group, but  $P$  value achieved insignificance. After EMF exposure for 6 h, there was statistically significantly increase in mtDNA copy number compared with the control group (**Figure 2**).

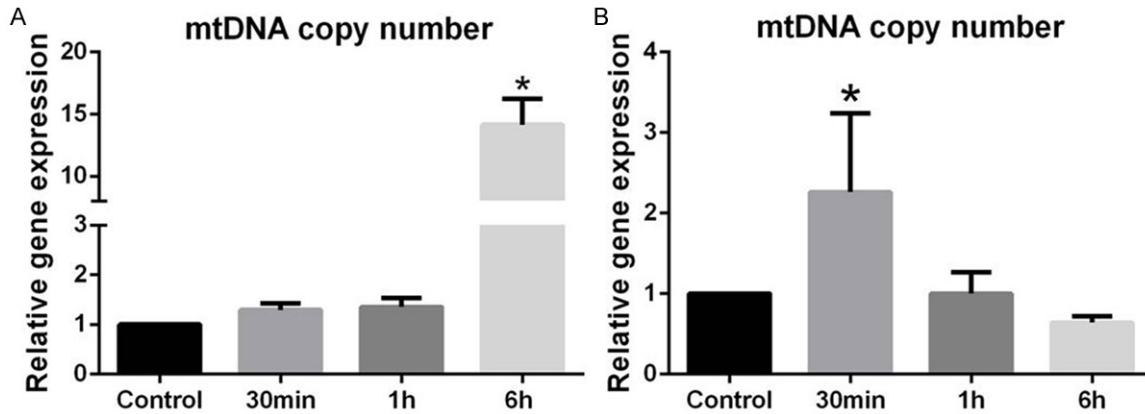
#### Effects of EMF on mtDNA copy number during MSCs differentiation

To determine whether 50 Hz EMF had effects on mtDNA copy number during MSCs differentiation, we chose two kinds of differentiation states of MSCs to analyze (**Figure 3**). After osteoblastic induction for 3 days, EMF treatment could increase mtDNA copy number which was in a time-dependent manner, but there was statistically insignificantly different after EMF exposure for 30 min, 1 h, except for 6 h, compared to the control groups. After neuronal induction for 3 days, mtDNA copy number of MSCs after EMF exposure for 30 min was significantly increased compared to the control group, then a significantly gradually decrease in mtDNA content was observed after EMF exposure for 1 h, 6 h.

### Discussion

In the present study, we demonstrated that 50 Hz EMF could affect mtDNA copy number of MSCs with different states, but the influences of EMF had different patterns. A transitory decrease in mtDNA content was observed during early osteoblastic differentiation, which was

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**Figure 3.** Effects of 50 Hz EMF on mtDNA content of MSCs with osteoblastic differentiation state (A) or neuronal differentiation state (B) for 30 min, 1 h or 6 h. MSCs after osteoblastic or neuronal induction were treated with EMF exposure. Total DNA was extracted and the mtDNA content was determined by QPCR and expressed as a fold-change from the level in the control group. The experiment was performed in triplicate. Data were mean  $\pm$  SD.

different from that during neuronal differentiation. We also found that 50 Hz EMF increased mtDNA copy number of MSCs in a time-dependent manner with the pluripotent state or during osteoblastic differentiation. During neuronal differentiation, mtDNA replication was enhanced after EMF exposure for 30 min, then a gradual decrease in mtDNA content was observed after EMF exposure for 1 h and 6 h.

According to the previous report [20], a low amount of mtDNA copies had been detected in undifferentiated embryonic stem cells, while this number increased upon differentiation. Furthermore, mtDNA replication increased during stem cells differentiation, but a transitory decrease was observed during the first days of differentiation [21, 22]. Similar to the results from Ye *et al.*'s experiment [21], we also found that a decrease in mtDNA copy number was observed during the early osteoblastic differentiation of MSCs. However, we found that mtDNA copy number was increased during the neuronal differentiation of MSCs. As we know, it was spent less time to induce neuronal differentiation than that in the process of inducing osteoblastic differentiation [23, 24]. Probably due to this, inducing neuronal differentiation for 3 days might be at later stage so that we did not find out a transitory decrease in mtDNA content.

Many studies confirmed that EMF affected proliferation or differentiation of MSCs [23]. Our results in this study showed that 50 Hz EMF increased mtDNA copy number of MSCs with

the pluripotent state in a time-dependent manner, meaning that 50 Hz EMF exposure promoted MSCs in a stress which was conducive to cells differentiation. In the previous study [11], we pointed out that 50 Hz EMF increased the percentage of MSCs at the G1 phase of the cell cycle in a time-dependent manner. Interestingly, when MSCs were exposed to 50 Hz EMF for 4 h/d for 3 days [25], EMF still promoted the cells proliferation. Following the above studies, we suggested that increasing in mtDNA content made MSCs more sensitization to their microenvironment and easy differentiation, but mitochondria function seemed to be tightly controlled in pluripotent cells unless their microenvironment changed [26].

Some reports demonstrated that EMF promoted osteogenic differentiation of MSCs [27, 28]. And excessive mitochondrial biogenesis could result in defective maintenance of the stem cells pool [26]. The results that EMF increased mtDNA copy number replied that EMF exposure could promoted the osteogenic differentiation of MSCs. Furthermore, our results showed that EMF induce osteoblastic differentiation in a time-dependent manner. Thus, we suggested that continual EMF exposure might be benefit for osteogenic differentiation of MSCs.

Several studies suggested that EMF induced ROS production and altered intracellular ROS concentration [12]. General speaking, a role in stemness and differentiation of stem cells was attributed to ROS [1], and ROS production was not restricted to cellular proliferation and dif-

ferentiation, but also phagocytic cells [13]. Excessive ROS accumulation could result in cells apoptosis. Evidence confirmed that EMF induced neural differentiation of MSCs was associated with ROS [12]. Though 50 Hz EMF with 60 min daily stimulation for 12 days could facilitate the induction of MSCs and differentiate into functional neurons [17], it had been also demonstrated that continual EMF exposure induced neural differentiation of MSCs in recent studies [29, 30]. Those evidences implied that continual EMF exposure did not cause excessive ROS accumulation. Intriguingly, reports showed that a certain level of ROS in mitochondrial can promote mtDNA replication [31]. However, our results in present study showed that EMF exposure resulted in a gradual decrease of mtDNA replication during neural differentiation and was time-dependent. Thus, we need more experiments to discuss whether continual EMF exposure decrease mtDNA replication though reducing ROS generation and its mechanism.

Many researchers suggested that EMF affected numerous biological functions within a certain range of low frequencies as well as low amplitudes [28, 29, 32], and EMF could enhance the differentiation process according to the experimental conditions. In the study, we also noted that EMF had different influences on MSCs mtDNA replication during inducing osteogenic differentiation or neural differentiation. So, using EMF treatment as a strategy for effective MSCs differentiation should meditate the optimal conditions and parameters [23].

In conclusion, for the first time we demonstrated that 50 Hz EMF affected the mtDNA copy number of MSCs with different states. The mitochondrial were proposed as a potential target of EMF on MSCs, but the detailed molecular mechanisms are not fully understood.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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