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Electromagnetic field exposed stem cells repaired Parkinson's disease symptoms in a rat model

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► ***Original article***

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ABSTRACT

Background: Some growth factors and electromagnetic fields (EMFs) are capable to differentiate bone marrow mesenchymal stem cells (BMMSCs) into neural cells. EMF may induce BMMSCs to differentiate into dopaminergic (DA) neurons. Our aim was to analyze the influence of EMF on BMMSCs in the treatment of rat models of Parkinson's disease. ***Materials and Methods***: BMMSCs were extracted from the rat's hind limbs and incubated in a cell-cultured CO_2 incubator. After the third passage, the BMMSCs were exposed to sinusoidal and square waveform EMF (400 µT, 75 Hz, 1 h/ day - 1 week or 7 h/1 day) and injected into the substantia nigra region of Parkinson rats. ***Results***: The results confirmed an increased number of TH+ neurons, a reduction of activated astrocytes, and an improvement in locomotor activity (Pole test) of sinusoidal EMF groups. ***Conclusion***: We presented a low-frequency sinusoidal EMF that increased BMMSCs' differentiation into DA neurons. The results indicated that injection of BMMSC exposed to sinusoidal 75 Hz EMF may increase TH+ cells in SNpc and motor coordination activity in the rat model of Parkinson's disease.

INTRODUCTION

Parkinson's disease (PD) is a neural system senile disorder that is accompanied by the diminishing of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) of the midbrain [1]. Dopamine medication is an effective pharmacologic method, but alternative treatments such as neuron transplantation are required in the later stages. Today, cell therapy would be an innovative treatment for PD. The tendency of researchers has concerned with the ability of the stem cells into the DA neurons differentiation [2]. Mesenchymal stem cells derive from bone marrow and can be modify into various forms of cells, like DA neurons. As a result, mesenchymal stem cells were used to reduce the symptoms of Parkinson's disease in animals [3]. Biochemical variables may influence the development of bone marrow mesenchymal stem cells (BMMSCs) into neural cells. The survival, proliferation, and differentiation of BMMSCs into neurons are essential factors for neurodegenerative disorder recovery. Earlier investigations had shown the effect of electromagnetic fields (EMFs) on the BMMSCs' differentiation into special varieties of neurons; and noninvasive treatment of EMF (50 Hz) could promote rat BMMSC differentiation into neurons [4]. Other results suggest that sinusoidal EMF (50, 60, or 100 Hz) induces neural differentiation in human BMMSCs [5, 6]. In addition, a few researchers interpreted the effects of EMF on the differentiation of BMMSCs in the treatment of Parkinson's disease. In agreement, Jadidi and collaborators reported that low intensity (400 µT) EMF might predispose to the differentiation of BMMSCs into DA neurons. The increment of BMMSCs' differentiation into astrocyte cells was related to the electrical frequency, and the highest differentiation was shown at 75 Hz EMF [7]. In opposition, other researchers have demonstrated that exposure to radiofrequency EMF decreases dopamine neuron numbers [8], shrinkage of pyramidal neurons, vacuolation of neurons and glial cells, and Purkinje cell reduction [9]. Compared with chemically induced differentiation, EMF radiation had better temporal control of the BMMSCs. Hence, EMF exposure would be operating in a time-dependent manner [10].

Accordingly, we chose to investigate the impact of EMF exposure on the viability and action of differentiated BMMSCs in the therapy of Parkinson's disease in this study. We believe that low-intensity, low-frequency (400 µT, 75 Hz) EMF [7] is the optimum type of electrical stimulation for BMMSC development because it causes the least harm to the cells. In a rat model of Parkinson's disease, this study used a low-threat EMF (400 µT, 75 Hz) to drive BMMSCs to develop into DA neurons, an increase in TH+ cells in the SNpc, and, as a result, an

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improvement in motor coordination activities.

MATERIALS AND METHODS

Experimental protocol

The animal breeding center of Semnan Medical University (Semnan, Iran) offered available 42 mature male Wistar rats (200±25 g, 6-8 weeks). All rats were kept in polycarbonate cages (4 rats/cage) with free access to tap water and food (light cycle of 12 hours, temperature 22±2°C, and humidity 50-70%). The rats were put into seven trial groups (n=6), each with six animals. As a control, 3 µg of Rotenone was injected into the left SNpc in the first group (Parkinson's). The first treatment, or the second group, has access to culture media. The third group received BMMSCs (sham-exposed) by SNpc injection. The fourth to seventh groups were treated by EMF irradiated BMMSCs (400 µT, 75 Hz square and sinusoidal waveform, 1 h/day for 1 week, or 7 h/1 day). A one-week pre-and post-treatment behavioral exam (Pole test) was done to assess the rat's activity. After the completion of the pole test, the animal's brain was attained to perform the immunohistochemical (IHC) process, including Tyrosine Hydroxylase (TH) and glial fibrillary acidic protein (GFAP) staining.

Surgical procedure

To anesthetize the animals', Ketamine hydrochloride/ xylazine hydrochloride (100 mg/kg - 20 mg/kg, Sigma-Aldrich) were used. After the skull was exposes by a 2 cm skin incision, a single hole was drilled over the left side. For injection into the left SNpc, the following directions were used (47): AP = - 4.9 mm posterior to the bregma, ML = -1.6 mm lateral to the midline, DV = 8.2 mm vertical from the dura (Paxinos and Watson, 1998). DMSO (10%, Merck, 102952) dissolved Rotenone (3 mM, 5 µL/rat, 1 µl/min, R8875, Sigma-Aldrich) was injected into the left nigral system to attain unilateral lesion in Parkinson rats [11].

BMMSC culture

In this study, BMMSC was aseptically extracted from the hind limbs of adult male rats and seeded in PBS (Gibco, BI1038500). Centrifuge the suspension (1500 rpm, 10 minutes) and place the resulting cells in DMEM (1/043 g L-glutamine, Gibco, 11530556), 20% (Gibco, BI10270-106), and penicillin streptomycin (BioIdea) in a 25 cm^2 flasks at 37 ^oC and CO_2 5%. The medium was changed after 48 hours for BMMSC to grow to a 70-80% surface. Three passages were used to purify BMMSC. Non-adherent cells (such as adipocytes and fibroblasts) were removed and adherent cells were washed with PBS every 3 days. At this stage, BMMSC was stained with trypan blue (Gibco, BI1014) and 5×10^4 cells in a 3.5 cm dish were incubated for EMF exposure.

EMF exposure

The EMF exposure system consists of a pair of coils (300 turns, internal diameter of 16 cm) with a square Plexiglas frame that was positioned in a cell culture incubator (5% CO_2, 37 °C).

A square or sinusoidal current was provided by a signal generator (GFG-8019G, Goodwill, Taiwan) and a 35 W audio amplifier (ALFA, Navasaz, Iran) to generate a magnetic field in the Helmholtz coil. BMMSCs exposed to 75 Hz, 400 µT EMF (1 h/day 1 week or 7 h/1 day) in the center of the magnetic field, apart from the exposure system, were turned off in the sham-exposed group. The condition of the BMMSC group was equal to other groups, except for turning off the EMF. The induction magnetic field at the center of the coils was calculated by a Teslameter (EMF 827, Lutron, Taiwan).

DiI labeling

To trace the injected cells, BMMSCs were labeled with CM-DiI immunofluorescence staining (Molecular Probes, Invitrogen, USA). Cells with a concentration of 10^6 were incubated in 5µg DiI for 20 min at 37 °C in a 95% air per 5% CO_2, and 2×10^5 cells were injected into the SNpc. Since CMDiI is fluorescent, it can indicate the location and distribution of BMMSCs in SNpc [3].

Behavioral testing

Pole testing makes it possible to estimate SNpc operation and motor coordination. The device consists of a wooden stick covered with a gauze bandage (60 cm high, 1 cm diameter) and a wooden marble above the stick (1.5 cm diameter). The bar is fixed to the ground by a sturdy board. The experimental conditions were presented to the experimental rats one day before performing the exercises. The trial tests were conducted in two stages; the first started one week before the treatment, and the final round was carried out one week after the treatment. The animals were placed head down on the wooden bullet and the time taken to turn down and move to the ground was recorded. The mean of three performed tests was calculated for statistical analysis.

Immunohistochemical study

At the end of the behavioral experiment, four rats were anesthetized; the brains were removed from the skull; the blocks of the SNpc were prepared, and embedded in paraffin. An IHC study was conducted to conclude the DA nerve populations within SNpc. Coronal sections (10 µm) of four rats were prepared and placed in 10% methanol and H_2O_2 for 8 min. The samples were washed 3 times with Tris buffer (pH: 7.4), then settled in citrate buffer (pH: 7.6) at 98 °C for 11-16 min and rinsed with Tris (pH: 7.4) 3-5 times. Samples were quenched inside 10% normal goat serum, 0.3% Triton X-100 and 1% bovine serum albumin (BSA) for 2 h (25-27°C). The brain samples

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were incubated overnight with the primary antibodies Anti-Tyrosine Hydroxylase, (ab137869, 1:500), and Anti-GFAP (ab7260, 1:100) to evaluate BMMSC differentiation into DA neuron and astrocyte cells. Afterward, the sections were washed in PBS several times and stained with chromogen 1% DAB (PVP1000D) to visualize and analyze the TH immunoreactivity and GFAP-positive astrocytes. Subsequently, brain samples were mounted, dehydrated, cleared, and cover slipped. The numbers of stain cells per area (mm^2) were analyzed from three sections with NIH Image J software.

Statistical analysis

Statistical analysis was accomplished by computer software (SPSS16.0, USA). One-way ANOVA and Tukey *post hoc* tests were done to evaluate SNpc slice data and locomotor activity exams. Statistical significance was present at $P<0.05$.

RESULTS

CM-DiI labeling was performed to estimate the location and distribution of cells injected into the SNpc region. Labeled BMMSCs crossed the blood-brain barrier and are located in the SNpc of the injured brain. TH immunohistochemistry was used to assess the number of TH+ neurons in the SNpc of experimental groups to see if BMMSCs may aid with neurodegeneration in Rotenone-injected Parkinson's mice (figure 1). Immunostaining revealed that EMF injection significantly increased the number of TH+ neurons in these groups as compared to the rotenone -only group. The rate of TH+ neurons compared to contralateral SNpc was recorded as the percentage of TH+ neurons. The results indicated that Rotenone causes a decrease in TH+ cells and DMEM administration has not had a treatment effect (13±1.7% and 13±2.6% respectively). As illustrated in figure 2, treatment with BMMSCs significantly increased DA neurons in injured SNpc (52.3±2.5%), (P<0.05). Injection of BMMSCs exposed to square EMF rose the percent of TH positive cells (42.3±1.5% and 38±2.6%) compared to the control group, and these amounts were less than the BMMSC group. The results confirmed the maximum number of TH+ neurons in the SNpc regions of BMMSCs exposed to sinusoidal EMF groups (67.3±1.5% and 62.3±2.5%).

Photomicrograph of GFAP immunostaining indicated markedly decreased number of activated astrocytes in the SNpc regions of treated groups (figure 3). Activated astrocytes were regarded for calculation based on the GFAP (astrocyte and microglia) activation following Rotenone injection in experimental groups. The mean number of activated astrocytes was assessed and set against the contralateral SNpc. As illustrated in figure 4, immunofluorescence staining showed a considerable rise in the activated astrocytes in control and DMEM treated animals (32.6±4.7% and 29.6±1.5%). Interestingly, BMMSC injection into the Rotenone-treated animals significantly declined (P< 0.05) the number of activated astrocytes compared to the control group (12.6±0.6%). In agreement, the results confirmed a reduced number of activated astrocytes in the SNpc regions of BMMSCs exposed to sinusoidal EMF groups (14.3±3% and 13.6±2.8%). Although treatment with BMMSCs exposed to square EMF rose the percent of activated astrocytes (28.3±2.8% and 27.6±2%), these amounts were the same as in the control group. In figure 5, the BMMSC post-differentiation ratio of TH+/Asrocyte cells was presented. The highest TH+/Asrocyte ratio was shown in the groups of BMMSC, sinusoidal EMF 1h/7d, and sinusoidal EMF 7h (4.1, 4.7, and 4.6 respectively). Although this ratio in the square EMF exposed groups declined, the relative amount was enormously greater than that of the control group (1.5, 1.4, and 0.4 respectively).

Outcomes of the second round of locomotors' activity (Pole test) confirmed the increased descending time in the control (Rotenone) group (21.1±3.5 vs. 24.6±1.5 sec), but the average time was not significantly different between tests (P> 0.05). The result of the DMEM injection group was the same as the control. In addition, rats could occasionally preserve their balance on the wooden bullet. As shown in figure 6, the findings also indicated that descending time was reduced in treatment groups and the difference in time was significant (P< 0.01).

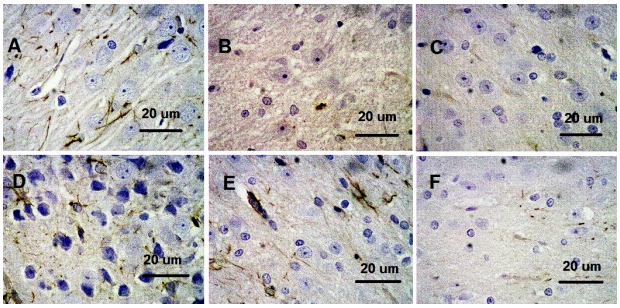

Figure 1. Expression of TH+ dopamine neurons in the SNpc of experimental groups: **(A)** Control, **(B)** BMMSC, **(C)** Sinusoidal 7d, **(D)** Sinusoidal 7h, **(E)** Square 7d, **(F)** Square 7h. Immunostaining indicated that EMF markedly increased TH+ neurons in the exposed groups.

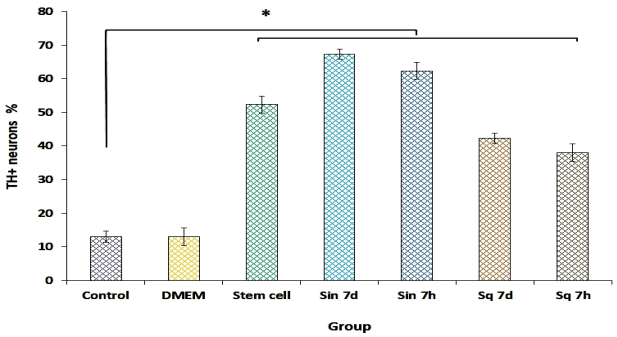

Figure 2. Quantification of TH+ neurons in the SNpc. The results confirmed maximum number of TH+ neurons in SNpc region of BMMSC exposed to sinusoidal EMF groups. The results represent the mean ± SD (* P<0.05). × 400.

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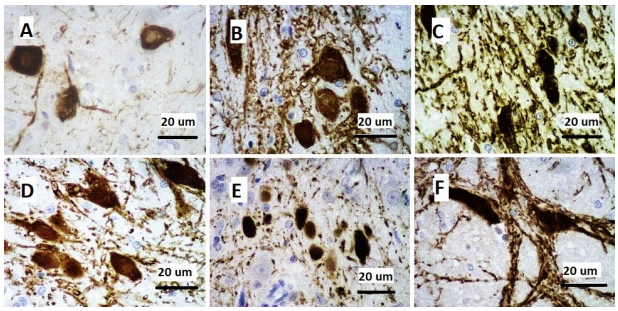

Figure 3. Representative light microphotographs of GFAP immunohistochemistry staining of SNpc in experimental groups: **(A)** Control, **(B)** BMMSC, **(C)** Sinusoidal 7d, **(D)** Sinusoidal 7h, **(E)** Square 7d, and **(F)** Square 7h. Immunostaining indicated markedly decreased number of activated astrocytes in the SNpc regions of treated groups.

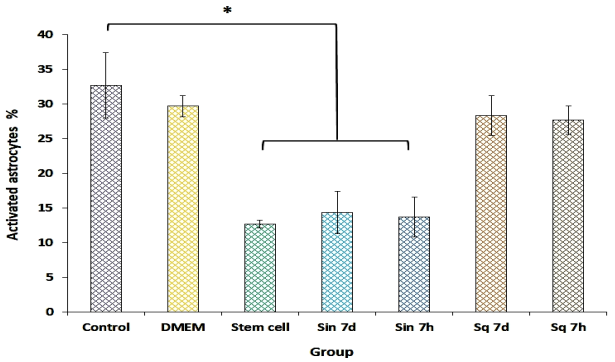

Figure 4. Quantification of activated astrocytes in the SNpc. The results confirmed minimum reaction of GFAP in the astrocyte cells of SNpc region of BMMSC and BMMSC exposed to sinusoidal EMF groups. The results represent the mean ± SD (* P<0.05). × 400.

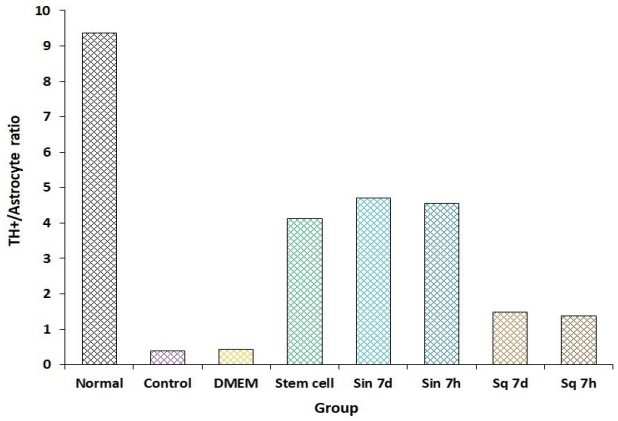

Figure 5. The ratio of TH+/Asrocyte cell density in SNpc of experimental groups. The most of TH+/Asrocyte ratio were shown in the groups of BMMSC and BMMSC exposed to sinusoidal EMF groups.

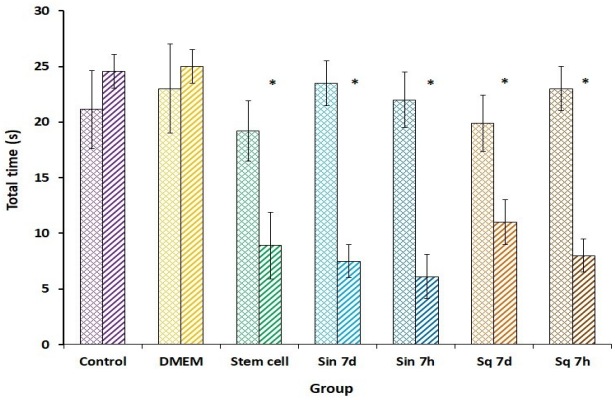

Figure 6. The locomotors activity (Pole test) of experimental groups one week pre and post treatment. The findings indicated that descending time was reduced in BMMSC and BMMSC exposed to EMF groups. The results represent the mean ± SD (* P<0.05).

DISCUSSION

Self-renewal competency and neurogenic potential (neurons, astrocytes, and oligodendrocytes) are the two specific capacities of BMMSCs. In addition, here we focused on the multi-potential BMMSCs, which, after EMF radiation, differentiated into DA neurons and were also successfully implanted in the SNpc of the rat brain. Naturally, most of the DA neurons are located in the SNpc of the midbrain, and this area participates in the motor activity and initiation of movement. The findings of this study indicated that Rotenone causes a decline of TH+ cells, and sinusoidal 75 Hz EMF exposure increased the number of TH+ neurons in the SNpc region of BMMSC groups. The motor coordination test improvement confirmed the result of this treatment in the rat model of PD. TH expression can indicate the survival and functional status of DA neurons.

In agreement, with the results of Bai *et al.* (50 Hz, 5 mT), Urnukhsaikhan *et al.* (60 Hz, 10 mT), Park *et al.* (50 Hz or 100 Hz, 1 mT), and Asadian *et al.* (75 Hz, 400 µT), enhancement of BMMSCs differentiation to neurons was reported [4-6, 12]. Furthermore, given that a noninvasive treatment of EMF (50 Hz, 400 µT) may increase DA neurons in the SNpc [7]. This is unlike the Kim et al. results, which showed that exposing the mice to RF-EMF (835 MHz) following MPTP damage decreased the number of striatum synaptic vesicles, DA neurons and impaired the recovery of locomotor activities [8]. In a study, methylene blue significantly enhanced the number of tyrosine hydroxylase-positive cells and decreased the rate of neuronal degeneration induced by Rotenone injection in the rat brain [13].

The present study also examined the relationship between EMF and activated astrocytes in the SNpc region. IHC staining confirmed maximum astrocyte activation in the Rotenone injected group compared with the sinusoidal 75 Hz EMF and BMMSCs groups. Contradictory result was represented in the experiment of Asadian *et al.*, that differentiation of the glial cell was increased after 75 Hz EMF radiation [12]. According to Barthelemy et al., data total GFAP was increased and, long-term memory was reduced after acute cerebral RF-EMF (900 MHz) exposure. This study suggested that RF-EMF prompted astrogliosis [14]. Furthermore, the proportion of GFAP-positive NSCs and GFAP mRNA expression did not differ

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substantially between the experimental groups exposed to low-frequency (5 mT) and high-intensity (2.5 T) EMF in Bai *et al.* study [15].

The first report of "reactive astrocyte" was presented during the 1970s after the discovery of glial fibrillary acidic protein (GFAP) [16]. The cooperation of astrocytes and neurons optimizes neural information processing, synaptic transmission, and neuronal activity. Astrogliosis saves the central nervous system from harmful processes [14]. Astrogliosis occurred with glial scars, proliferation, and migration of astrocytes in animal models of PD. The rate of astrogliosis is proportional to neural degeneration. Astrocyte increase and high GFAP-Immunoreactivity are characteristics of severe astrogliosis [17].

The main finding out of this study was that although the number of transplanted cells was the same, the ratio of TH+/Asrocyte cell density was not equal in the sinusoidal EMF exposed and square EMF exposed groups. Because of an increased number of TH+ neurons in BMMSCs treatment groups, the highest TH+/Asrocyte ratios were shown in the groups of sinusoidal EMF 1h/7d, sinusoidal EMF 7h, and then BMMSC alone (4.7, 4.6, and 4.1 respectively). This may be related to BMMSCs' differentiation into DA neurons and a decline in the astrogliosis process. Stimulation of transcription and protein expression may be affect by electron transport and imperil the hydrogen bonds of cellular macromolecules after EMF radiation. Therefore, indirect mechanisms because of genotoxic effects that follow the stimulation of reactive oxygen species (ROS). A minor quantity of ROS stimulates signaling cascades and participates in cell development and differentiation. Hence, EMF induction of stem cell differentiation supports its use in neural cell injuries [18]. Accordingly, EMF-activated BMMSCs increased the brain BDNF and led to increased tyrosine hydroxylase neurons in SNpc, which have good effects on PD treatment [7]. EMF describes a non-invasive treatment that can be used alone or in combination with conventional therapies for motor and non-motor symptoms of PD, but for style management of electrotherapy. From, different parameters (frequency, stimulus intensity, duration and pattern) are remarkable [19, 20]. However, these EMF parameters are different, and this would explain the inconsistencies. Hence, we cannot clarify the mechanism of the EMF effect on BMMSCs' differentiation. Moreover, the functions of the EMF on the PD are still unclear and need more investigation to attempt to determine the precise mechanisms.

CONCLUSION

We used a low-intensity EMF (400 μT) to test the ability of BMMSCs to differentiate into DA neurons. The results indicated that injection of BMMSC exposed to sinusoidal 75 Hz EMF may increase TH+ cells in SNpc and motor coordination activity in the rat model of PD. Further studies on the effects of EMF on BMMSC application in PD are recommended.

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***Authors' contributions*:** Taha Jadidi, carried out the experiments and data collection. Nader Asadian, helped with data collection and stem cell culture. Manouchehr Safari and Hamid Reza Sameni, cooperated as scientific advisers to behavioral tests and stem cell cultures. Vahid Semnani: analyzed and interpreted the data. Majid Jadidi, participated in experiment design, EMF exposure, and wrote the manuscript. All authors read and approved the final manuscript.

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