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Article *in* The Environmentalist · June 2009

DOI: 10.1007/s10669-008-9210-4

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DOI 10.1007/s10669-008-9210-4

Investigation of the effects of static magnetic field on apoptosis in bone marrow stem cells of rat

Zeinab Tavasoli · Parviz Abdolmaleki · Seyed Javad Mowla · Faezeh Ghanati · Amir Sabet Sarvestani

Published online: 11 February 2009

Abstract This research was carried out to evaluate the influence of static magnetic field on the rate of apoptosis in bone marrow stem cells (BMSCs) of rats. Extracted cells were suspended in αMEM as a culture media for 48 h. After that, cells were exposed to 15 mT static magnetic field (SMF) for 5 h, incessantly with or without $FeCl_2$. The rate of apoptosis was then assessed via flow cytometery. The results showed that either treatment with $FeCl_2$ or exposure to SMF enhanced the rate of apoptotic cells. Moreover, cells that were treated simultaneously with $FeCl_2$ and SMF have higher rate of apoptosis. An increase in apoptosis by 26.5% was induced by SMF alone and an increase in apoptosis by 28.2% was induced by a combination of $FeCl_2$ and SMF, compared to their corresponding controls. The results recommended that the effects of SMF on apoptosis may be related to increment of the number of free radicals in the cells.

Keywords Stem cell · Apoptosis · Static magnetic field · $FeCl_2$ · Flow cytometry

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1 Introduction

Magnetic fields (MFs) are widely distributed in the environment and their effects are increasing. Much concerns rose about the possible biological effects in a large variety of cellular functions. However, the previous reports are rather controversial and the exact mechanism is still unclear (Lacy-Hubert et al. 1998; Repacholi and Greenebaum 1999; Yoshikawa et al. 2000; Jajte et al. 2001). A possible mechanism that can explain many of the observed biological effects of magnetic fields is free radical production. Magnetic fields can increase the life time of radicals and these radicals are accessed for more radical–molecule interactions (Hulbert and Metcalfe 1998). As a result, more interaction would be done between free radicals and cellular components. Abnormalities in gene expression, damage of proteins, changes in the activity of certain enzymes, effects on membrane structure, cell growth, and cell death are the consequents of oxidative stress (Mohtat et al. 1998).

There is a balance between the processes of proliferation and apoptosis in normal cells (Guo and Hay 1999). Disruption of this balance due to exposure to the physical or chemical stimulators such as MF or iron may result in either too much or too little cell numbers, which is observed in cancers and degenerative diseases, respectively (Dini and Abbro 2005). Iron is a ferromagnetic element that could be affected by magnetic fields. Iron is also responsible for the production of hydroxyl radical (OH) through the Fenton and Heber–Weiss reaction (Dat et al. 2000). Increment of the amount of iron in the cells can alter the rate of apoptosis through the antioxidant processes (Jajte and Grzegorczyk 2002).

In an attempt to consider the possible potential usefulness of applying MF in cancer treatment, we intended to

use bone marrow stem cells (BMSCs) as a biological target for static magnetic field (SMF). This would be helpful if a pro-apoptotic role for MF is established through experiments on stem cells that are recently introduced as the origin of cancer (Pardal et al. 2003). Briefly, it has been reported that tumors are heterogeneous in terms of cell phenotype and proliferating potential, even in cases in which all cells are derived from a single clone. Although ongoing mutagenesis can partially explain this heterogeneity, it also seems that some tumors arise from small populations of "cancer stem" cells that give rise to phenotypically diverse cancer cells, with less proliferative potential. These cancer stem cells are likely to arise from mutations that dis-regulate normal stem cell self-renewal (Ulloa-Montoya et al. 2005). The characterization of BMSCs as an adult stem cell is then essential before any implementation in clinical application.

In this study we exposed the BMSCs as a normal adult stem cell with the ability to mutate into the CSCs into the SMF to clarify whether the SMF is a pro- or anti-apoptosis stimulator on BMSCs. Furthermore this characterization may improve the diagnostic and therapeutic methods in cancer treatment through clarifying the general knowledge about mechanisms of the responses of the BMSCs to SMF.

2 Material and methods

2.1 Isolation and implantation of stem cells

Eight to ten week-old rats (Razi institute, Tehran, Iran) were killed and tibias and femurs were dissected out and placed in PBS on ice. Under sterile conditions two ends were cut with scissors, and then marrow was flushed out using a syringe with a no. 26 gauge needle filled with 10 ml αMEM (Invitrogen, UK). The extracted cells were seeded in αMEM supplemented with 20% fetal bovine serum (FBS; Invitrogen, UK), 100 U/ml penicillin, 100 μg/ml streptomycin and 25 ng/ml Amphotericin B in a 75 cm^2 flask and incubated at 37°C with 5% humidified CO_2. Non-adherent cells were eliminated by changing the medium on the next day and the adherent cells were allowed to propagate. Confluent BMSCs were passaged and plated out at 1:4 or 1:5 dilutions every 5–6 days using 0.25% Trypsin and 1 mM EDTA. The cells were frozen in αMEM with 30% FBS and 5% DMSO in liquid nitrogen.

As mentioned above, αMEM (Invitrogen) with 20% fetal bovina serum (FBS) (Invitrogen) was culture media for these cells. Culture media of these cells would be replaced after 48 h. Since the stem cells are adhesive, their old culture media which contains dead cells were outpoured for passing them and the live cells were separated with 1 ml Trypsin + EDTA. Following this, trypsin was neutralized with serum that is in culture media and the cells were transmitted into the new flasks. After three or four passages stem cells are ready for treatment.

2.2 Treatment of cells

2.2.1 Magnetic field generator

Exposure to MF was performed by a locally designed MF generator. The electrical power was provided using a 220 V AC power supply equipped with variable transformer as well as a single-phase full-wave rectifier. The maximum power and passing current were 1 kW and 50 A DC, respectively. This system was designed to generate MF in the range of 0.5–30 mT. It consisted of two coils (each with 3,000 turns of 3 mm copper wire) on a U-shaped laminated iron core (to prevent eddy current losses). Using two vertical connectors, the arms of the U-shaped iron core were terminated in four circular iron plates covered with thin layer of nickel (each 23 mm thickness, 260 cm in diameter).

A water circulation system around the coils was employed to avoid the increase in temperature. Without this system, the temperature of the coils would increase, after the apparatus works for 10 min, from 25 to 40°C. Applying the water circulation system helped us to use the apparatus for long exposures (5 h) as well as to keep up the maintenance of the apparatus. The temperature between the circular iron plates, where the samples were located, was measured and was almost the same as other parts of the room, e.g., the location site of the control cells, ±1°C.

Presence of any pulsation in the current from rectifier into the MF generating apparatus, was tested by an oscilloscope (40 MHz, model 8040, Leader, Japan) and a pulsating frequency of 50 Hz with a range of voltage variation about ±1 V, was shown. The presence of this pulsating frequency may be related to the shortcoming of the single-phase full-wave rectifier, which provides a ripple voltage around 5%. This small ripple voltage suggests that the generated MF can be considered highly homogeneous.

Calibration of the system as well as tests for the accuracy and uniformity of the MFs were performed by using a teslameter (13610.93, PHYWE, Germany) with a probe type of Hall Sound. The accuracy of the system was ±0.1% for MF and the range of measurements was 3 mT–30 mT.

2.2.2 Treatment of cells

Twenty-four hours after the last renewal of culture media, the flasks containing at least 10^6 MSCs treatment of BMSCs of rats were accomplished.

In this study, the effect of SMF and $FeCl_2$ were investigated simultaneously. In the time of treatment, cells were

divided into four groups. First group of cells were exposed to the SMF alone. Second group was treated with $FeCl_2$. Third group was exposed to both of treatments and fourth group was control, without any treatments. In this research, the quantity of $FeCl_2$ was 20 μg/ml and intensity of SMF was 15 mT for 5 h without any interruption.

2.2.3 *Apoptosis detection by flow cytometry*

Flow cytometry is one of the methods that are utilized to detect apoptosis. A flow cytometer can analyze too many individual cells rapidly. The way by which the apoptotic cells were distinguished from non-apoptotic cells is that apoptotic cells have fragmented DNA and their attachment to specific fluorocromes such as propidium iodide (PI) was diminished (Alvaro and Marta 2000). Stored solution of propidium iodide was prepared by dissolving 1 mg of its powder in 1 ml phosphate buffer saline (PBS). This solution is constant in dark place at 4°C for several months. Twenty microliters from this stored solution plus 1 μl triton ×100 and 20 μl Ribonuclease A (RNase A) is the final cocktail that is used for staining the cells. Before staining, cells were separated from the flasks using Tripsin. Afterward, cells were centrifuged at 200 g for 6 min. In order to fix the cells, 0.5 ml PBS and 4.5 ml cool ethanol were added to the remaining cells. In the time of staining, the cells were centrifuged and 1 ml final cocktail was added to them.

Apoptosis detection was executed using a LSR II flow cytometer (Becton Dickinson). PI fluorescence was collected with a 575/25 nm band pass filter, orange–red fluorescence (FL2), after linear amplification. Flow cytometric data was stored according to a standard format, the flow cytometry standard (FCS) format. According to the FCS standard, a data storage file includes a description of the sample acquired, the instrument on which the data was collected, the data set, and the results of data analysis. By the Cell Quest program histogram plots were created. For each measurement, data from 10,000 single cell events were collected. An important principle of flow cytometry data analysis is gating in which single cells were collected. According to physical individualities cells have traditionally been gated. For example, subcellular debris and clumps can be distinguished from single cells by size, estimated by forward scatter. Cell doublets and aggregates were gated out using a two parameter dot plot of FL2-Area versus FL2-Width. Cell cycle histograms were analyzed using Win MDI 2.9. Based on this program, only cell cycles with low variation coefficient ($CV < 0.05$) were used for further statistical analysis (Hawley and Hawley 2004).

For detection of apoptosis, histogram was drawn based on the DNA contents and cell counting. When DNA contents were decreased in apoptotic cells, PI entered the cells and a pick was created in histogram before $G_0 - G_1$ (Fig. 1). As a result, the over lapping of apoptotic and non-apoptotic cells was diminished.

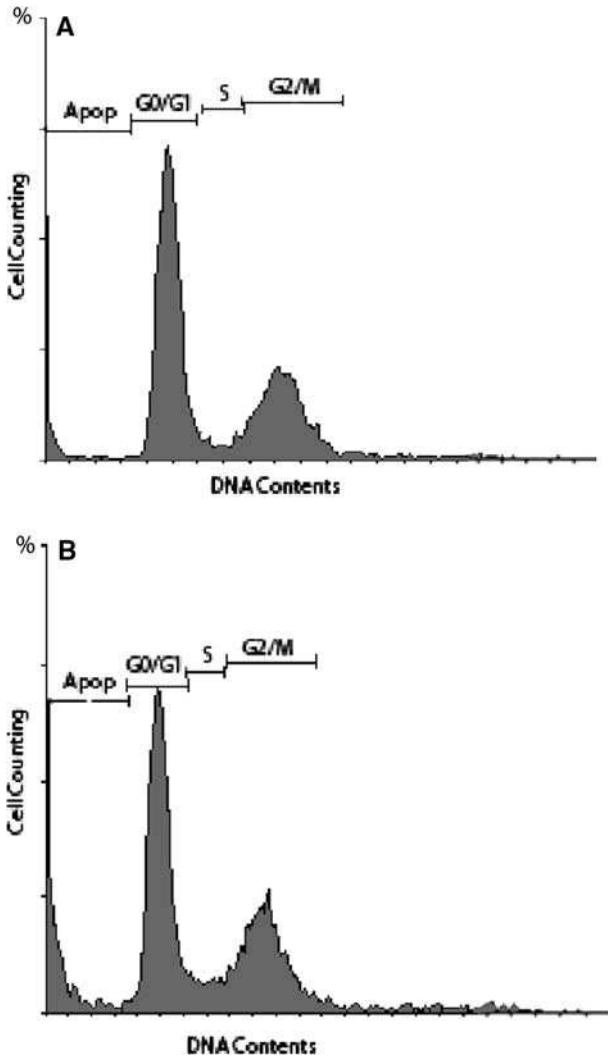

Fig. 1 Histogram of cell cycle phases for BMSCs. **a** Cells were treated only with $FeCl_2$. **b** Cells were treated with SMF and $FeCl_2$. Apoptotic cells were shown in the pick before $G_0 - G_1$

3 Results

In the present study, the results of flow cytometry demonstrate that exposure of BMSCs to SMF leads to an increase in their apoptosis. The rate of apoptosis in control was 29.88% while the rate of apoptosis in cells treated with SMF was 56.38% which showed 26.5% difference due to exposure to SMF. Similarly, the rate of apoptosis in cells treated with SMF plus $FeCl_2$ was 58.09% which show 19.21% differences compared to the corresponding controls (38.88%) (Fig. 2). The results of this investigation were statistically analyzed by SPSS software. A difference was considered statistically significant when $P < 0.05$.

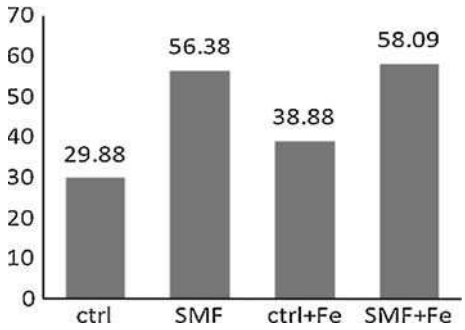

Fig. 2 The percentage of apoptotic stem cells in 15 mT SMF for 5 h

4 Discussion

Epidemiological and experimental data have attracted attention to the biological effects of MF. Primary action of MF in biological systems is the induction of electrical charges and currents (Roy and Repacholi 2005). One of the major molecular effects of MFs is their influence on nuclear spins of paramagnetic molecules. This mechanism plays an important role when in the course of a chemical reaction, the chemical bond is disrupted, and two molecules with unpaired electrons are formed (a pair of radicals). Depending on their spin orientations, radical recombination or diffusion, and formation of free radicals (e.g., oxygen radicals) may take place (Kula et al. 2002; Sobczak et al. 2002). The radical pairs may be affected for a long and continuous time interval by the MF (Zmyslony et al. 2000). This may extend the lifetime of the free radical and its potential damage could be exaggerated.

The present study initially indicated that exposure to SMF (15 mT, 5 h) induced apoptosis in BMSCs of rat. Moreover, iron ion augmented the effect of SMF in these cells. The rate of apoptosis increased from 29.88 ± 12.96 to 56.38 ± 31.87 in 15 mT SMF treatment. This means that apoptosis increased by 26.5% under SMF compared with its control samples. Also the increment of apoptosis was shown when BMSCs were treated with iron ion and SMF compared with cells that were treated only with SMF.

Previous investigations have shown that alteration in the amount of iron ion in cells can affect the rate of apoptosis (Jajte and Grzegorczyk 2002). The increment of iron in cell leads to increment of hydroxyl free radicals via Fenton reaction (Dat et al. 2000).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$$

In this investigation, BMSCs were treated with SMF and iron ion, simultaneously. The results demonstrated an increment by 28.2% compared with the control samples. Therefore, iron ion can amplify the effects of SMF on apoptosis.

Free radicals, directly and indirectly, participate in the operation of apoptosis and necrosis. Studies show that 0.25 mT SMF can operate apoptosis in fibroblast cells of rat (Blumenthal and Ricci 1997). Also in another research, 7 mT SMF in the presence of iron ion can cause cell death (apoptosis and necrosis) that is justified with the influence of magnetic field on free radicals (Jajte and Grzegorczyk 2002).

Altogether, the results of this investigation demonstrate that SMF and iron ion can enlarge the rate of apoptosis in stem cells. Although the exact mechanism of this effect is still unknown, evidences of this research support the hypothesis that both SMF and iron can promote apoptosis with modification of the amount of free radicals and increment of their stability. Consequently, SMF might be useful to be applied in degenerating cancer stem cells that have recently been suggested to be the origin of cancer in cancer treatments (Pardal et al. 2003). This is justified because recent researches demonstrated that only a small minority of tumor cells were able to proliferate extensively (Reya et al. 2001). This gave rise to the idea that malignant tumors are comprised of both cancer stem cells (CSCs), which have great proliferative potential, as well as more differentiated cancer cells with limited proliferative potential. On the other hand, the conventional cancer treatments were mostly focused to kill the tumor cells instead of the CSCs; therefore the CSCs as the origin of cancer were left untouched during the treatment. This may be the reason of the recurrence because although the tumor shrinks following the treatment, it grows back. Possibly new drugs or treatments should be designed to kill the CSCs initially instead of tumor cells (Pardal et al. 2003). To get close to this idea, characterization of CSCs when exposed to the physical and/or chemical stimulators such as SMF or $FeCl_2$ has great importance. Consequently, applying the principles of stem-cell biology in conjunction with SMF may lead to an understanding of the influence of SMF in tumor development and progression so that it may improve cancer therapy by playing a pro-apoptosis role.

In conclusion, based on the primary results of this investigation, SMF alone, and in conjunction with $FeCl_2$ may be used as a therapeutic method to treat cancer, because of its ability to induce apoptosis in BMSCs of rat. However, further investigations should be performed to apply the SMF in clinical assessments to improve the cancer treatments.

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