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Research Article

Static Magnetic Field Effect on Differentiation in Human Mesenchymal Stem Cells

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Abstract

Stem cell therapy offers great hope for patients suffering from diseases for which there is currently no cure. The aim of this study is to investigate the effects of static magnetic field on cellular differentiation and evaluates 18milli Tesla field effect on human umbilical cord-derived mesenchymal stem cells neural differentiation which makes them favorable candidates for some clinical applications.

Human cord-derived mesenchymal stem cells were isolated and expanded based on the previously reported experimental conditions and neural differentiation was induced in the presence of magnetic field for the treated samples. Differentiation induction medium was made of a-modified minimum essential medium containing retinoic acid (0.5µM/l) which was replaced with a favorable medium for neural cell growth After exposure made of DMEM F12 (#12660-012) supplemented with Serum-free N2/B27, L-glutamine (2mM), bfGf (20µg/l) and Fetal Bovine serum 5% respectively. All samples were cultured for 3 weeks and were finally observed using light and fluorescence microscopes and examined for gene expression control.

Our data showed that static magnetic field exposure causes *Sox-2*, *Nanong*, and *Oct-4* gene expression decline, conversely *hEAG*-1 and Nestin genes expression increase after three weeks post-exposure culture time. The effects became dramatic in the presence of retinoic acid suggesting the auxiliary differentiation induction effect of static magnetic field.

Stem cell showed that physical inducers just like SMF in moderate intensities along with the chemical ones including retinoic acid can enhance HMSCs neural differentiation to produce neural-like cell lineages.

Keywords: Stem cells; Magnetic field; Wharton's Jelly; *hEAG*-1; Nestin; Neural differentiation

Abbreviations

HCMSCs: Human Cord Derived Mesenchymal Stem Cells; BM: Bone Marrow; mT: milli Tesla; SMF: Static Magnetic Field; MFs: Magnetic Fields; RA: Retinoic Acid; WJ: Wharton's Jelly; ICC: Immunocytochemistry

Introduction

Stem cells are present in all human organisms and capable of reproducing themselves or switching to become more specialized cells in human body such as cells in brain, heart, muscles and kidney to repair damaged tissue [1]. Recent advancements in basic and clinical research on bone marrow, embryonic, fetal, amniotic, umbilical cord, and adult stem cells have revealed multiple possibilities for stem cell new potential therapeutic uses which emerge as new powerful tools for tissue engineering and regenerative medicine [2].

Mesenchymal Stem Cells (MSCs) are pluripotent progenitor cells because of their differentiation and immune-suppressive properties that make them appropriate for transplantation and clinical use [3]. These cells retain the ability of developing into primary germ cell layers of the early embryo, which can differentiate into cells of different connective tissue lineages such as bone, cartilage, muscle and fat of the adult body. A large number of animal transplantation studies showed that MSCs must be differentiated into the residing tissue to repair damaged cells caused by trauma or disease, and partially restore its normal function [4]. Since MSCs have been investigated to grow and differentiate toward a pattern of multilineage differentiation potential to produce different cell phenotypes throughout their life, they are emerging as powerful tools for tissue engineering [5].

Static Magnetic Field (SMF) interaction with the living organisms has been a rapidly growing field of investigation in the recent decades. Some reports clarified that transplantation of bone marrow cells of magnetic-field-exposed mice led to increased numbers of spleen cell colonies (CFU-S 7d) in conditioned recipient mice [6].

While Magnetic Field therapeutic uses for healing human diseases have been stablished from a long time ago, its new application in cancer tissue treatment by altering cell fate leading to malignant tissue differentiation is a novel approach in this area [7]. Differentiation therapy which has been recently characterized as a potentially less toxic approach compared to chemotherapy uses some agents just like Retinoic Acid (RA) to induce cancer cell differentiation [8,9].

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Figure 1: SMF generator equipped with a biological Incubator for providing standard cell culture conditions.

On the other hand, SMF can also help differentiation induction in stem cells, so this physical inducer has been employed for its anti-proliferative function through differentiation-dependent apoptosis among the malignant cells. SMF differentiation effect has also been stablished by many of the recent studies such as chondrogenic differentiation of adult human bone marrow-derived stromal cells [10].

Mardedzaik et al. showed the SMF enhancing effects on osteogenic properties of Human Adipose-Derived Mesenchymal Stem Cells (HAMSCs). SMF effect has also been noted to accelerate differentiation in human stem cells [11]. The results clearly suggested a direct influence of SMF on the osteogenic differentiation potential of HASCs. These results provide key insights into the role of SMF in changing cell fate [12].

This study has also pointed to some of the already stablished stem cell studies and tries finding new strategies in the field of SMF differentiation effects to clarify some of its potential uses for regenerative medicine research. In particular we have investigated whether 18milli Tesla (mT) exposure MFcan enhance chemically-induced neural differentiation or not.

Materials and Methods

Static magnetic field exposure

Exposure to MF was performed using a locally designed Static Magnetic Field (SMF) generator. The magnetic field generator consisted of two coils and a DC switching power supply. The coils were built from a 3.0mm diameter wire, and resistant to heat up to 200°C. Coils had a total resistance of 3ohm (Ω) and the inductance of 2 Henry. These two coils guided the magnetic field through two iron blades with the height of 1 meter and the cross section of 10cm. The electrical power was provided using a 220V/AC power supply equipped with a variable transformer as well as a single-phase full-wave rectifier. The switching power supply could apply a DC voltage up to 50Volt and a current up to 16Ampere to the coil for moderate intensity static magnetic field generation as needed.

In order to cool off the system, a gas chiller with optimum control on temperature was used. This cooling system consisted of an evaporator, an engine, a condenser, and refrigerant gas (Figure 1).

The evaporator covered the outer surface of the coils. This enables the system to effectively cool the system down. The gas introduced to this motor was of R12 kind. The field between the iron blades was measured by a 13610.93 PHYWE (Gottingen, Germany) Teslameter. Presence of any pulsation in the current from rectifier into the SMF generating apparatus, was tested by anoscilloscope (8040, Leader Electronics Co., Yokohama, Japan). However, the ripple voltage of the field produced by the system was simulated using the CST STUDIO 2011 software.

The profile of field emission in coils calibration of the system as well as tests for the accuracy and uniformity of the SMF were performed by a Teslameter (13610.93, PHYWE, Gottingen, Germany) and also was calculated with Complete Technology for 3D Simulation CST STUDIO 2011 software (http://www.CST.com) for the best site selection. Static Magnetic Field (SMF) generator was also equipped with a locally designed incubator placed between U shape iron arms to provide standard cell culture conditions within the chamber of SMF generator providing homogenous 18mT SMF exposure.

The control samples were only exposed to geomagnetic field of earth which was 0.047mT in our lab. It would be impressive if we could but the measurement was performed by Institute of Geophysics, University of Tehran, Iran.

Isolation of Human Mesenchymal Stem Cells (HMSCs)

Cord samples collected at delivery from newborns with their parents' consent and were donated to Royan Cord Blood Bank, (Research and Development section, Tehran, Iran). This study has been conducted according to Declaration of Helsinki principles. All experiments were performed according to the stablished protocols provided by the Ethics Committees of Tarbiat Modares University and Royan Stem Cell Technology Company (Cord Blood Bank) Research Center, and the new born parents' written informed consent letters were collected prior to participation.

At the first step blood vessels were initially detached from the cords in order to extract Wharton's Jelly (WJ) which subsequently was treated with Collagenase Type IV 1% in PBS (Phosphate Buffered Saline) for 45min in the standard cell culture condition.

Wharton's Jelly (WJ) pieces were then discarded and the suspension was centrifuged (1500rpm, 5min, 20°C). After 45min of incubation the pellet was cultured in Low Glucose DMEM medium (Gibco—life technologies TM—Cat # 21885) supplemented with 15% FBS (Fetal Bovine Serum), 1% penicillin/streptomycin, and 1% L-Glutamin. HMSs were purificated and adhered to the bottom of the plate after 2 weeks.

Each experimental sample which was harvested and counted to provide70% confluent monolayer's contained about 1million cells in each cell culture flask with an area of 25cm^2.

Identification of HMSCs Immunofluorescence experiments showed the presence of surface antigens of mesenchymal stem cells(CD44, CD73, CD90, and CD105) and the absence of hematopoietic ones (CD133, CD45, and CD34).

The lake of HLA-DR surface antigen was also examined in the cord derived MSCs.

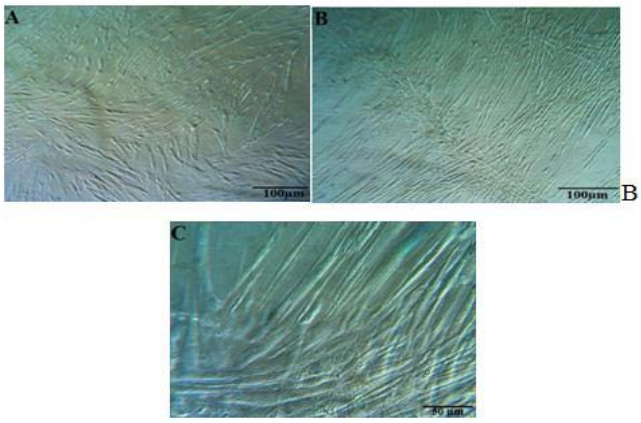

Figure 2: A: HMSCs in the differentiation medium control sample; B: After exposure, SMF-induced cell alignment is evident, "B" arrow indicates the magnetic field direction; C: SMF treated sample in the same culture medium (with an objective lens magnification of 40X).

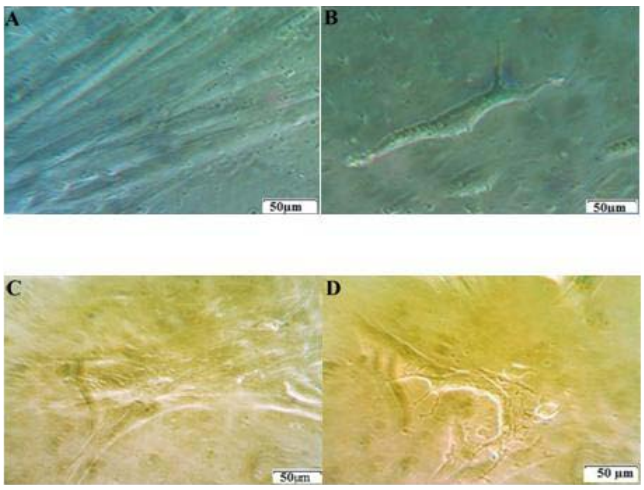

Figure 3: Light microscope photographs indicating SMF effect on HMSCs differentiation. A: Control sample with chemical (RA) induction and without SMF exposure after 2 weeks in neural tissue medium; B: The sample which has been exposed to 18 mT SMF for 6 hours after 2 weeks cultured in neural tissue medium; C: Control non-exposed sample after culturing for 3 weeks in the same culture medium; D: RA induced and SMF treated sample after 3 weeks in the same culture medium, oligodendrocyte-like morphology is evident in this photograph.

The potential of HMSCs for differentiation was investigated by providing the proper differentiation conditions in our laboratory in order to ensure that the samples are truly mesenchymal stem cells. Multi lineage differentiation potential of the cord extracted cells into bone, cartilage and fat was examined as reported in our previous study. This has also been described as one of the main characteristics of MSCs by other laboratories [13,14]. Cord-derived cells' triple differentiation ability was another evident in addition to surface antigen representation which proved that the extracted cells are MSCs as we reported in our previous study [15].

SMF treatment

Human Cord-Derived Mesenchymal Stem Cells (HCMSCs) were extracted, expanded *in vitro* and prepared for SMF exposure at the third passage, as well as the identical control samples. Cell cultures were harvested and counted to provide cultures at the 60% confluence for the experiments. Before each exposure, the SMF intensity was set using the Teslameter to make sure that no other magnetic appliance was working. Samples were exposed to SMF (18mT) for 8 hours while the control samples at the same time were placed inside another incubator in the isolation under standard cell culture conditions. Then all samples were cultured for three weeks.

Differentiation medium

Pervious to SMF exposure, samples were initially cultured in neural differentiation induction medium made of a-modified Minimum Essential Medium (a-MEM)/1 mM b-Mercaptoethanol, (bME) 10mM, a-MEM/Retinoic acid (RA) (0.5µM/l), and after exposure in a new experimental condition in which the RA was removed from the medium. Cell culture samples after SMF exposure along with the unexposed control samples were cultured for 3 weeks in Knockout DMEM F12 (#12660-012) supplemented with Serum-free N2/B27and L- glutamine (2mM), bfGf (20µg/l) and 5% Fetal Bovine Serum (FBS) respectively was known as a modified medium for neural cell growth [16]. All the treated and control samples were then cultured in the identical conditions for differentiation induction and post exposure culture time.

In our experiments all samples (the SMF treated and the corresponded control ones) were induced by Retinoic acid prior to exposure and cultured in the neural-cell fevered medium for three weeks after it. Then they were prepared for the next steps of experiences including gene expression control.

Immunocytochemistry (ICC)

Antibodies conjugated to fluorescent molecules are functional tools for finding definite proteins in cells, a method referred to as Immunocytochemistry (ICC). Cell cultures at the third passage were harvested and washed using PBS buffer then transferred to 4-well cell culture plates contained 3000/ml cells in each well. After 48 hours the medium was discarded and wells were washed three times by PBS. The washed samples left for 15 minutes to be dried in the room temperature and then fixed by acetone for 2 minutes and incubated at 4°C for one hour.

Human Nestin goat anti-mouse: 50µg/ml (Ebnesina research institute-Tehran, Iran) was then added to each well by overnight incubation at 4°C. Anti-mouse ATT conjugated secondary antibody 1/200 (Tween 0.1% in PBS) was added and samples were washed with PBS and studied by fluorescence microscope. At the end of the procedure Propidium Iodide (PI) staining was performed to visualize cell nuclei [17]. Fluorescence microscope photographs were then assessed using Imagej software to measure color percentages to perform data analysis [18].

Molecular tests

RNA extraction and cDNA synthesis: All samples were harvested and prepared for RNA extraction (Qiagene Rnasy mini kit #74104) and cDNAs synthesis performed using Fermentase Revert First Strand cDNA synthesis kit #K1622. Agarose gel Electrophoresis of PCR products revealed the gene transcription levels between samples.

Rt-PCR: Polymerase Chain Reaction (PCR) is a method that

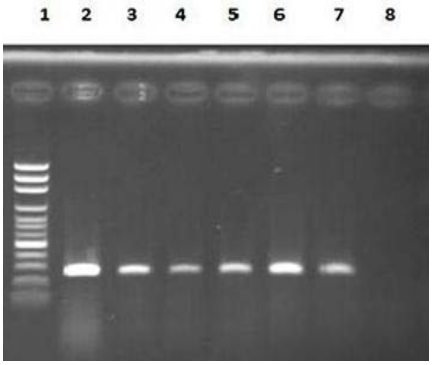

Figure 4: *Oct-4* PCR products agarose gel-electrophoresis photograph; 1: Ladder; 2: *GAPDH* (control+); 3: RA treated sample after 3 weeks; 4: SMF and RA treated samples after 3 weeks; 5: only SMF- treated samples after 3 weeks; 6: control sample without RA and SMF treatments after 3 weeks; 7: SMF and RA treated sample after 2 weeks; 8: control- (without *oct-4* primer).

allows exponential amplification of short DNA or RNA sequences of usually 100 to 600 bases length within a double stranded molecule using a pair of primers (up to 30 nucleotides) which are complementary to a determined sequence on each of the two strands of the macromolecule.

Reverse transcriptase-PCR (Rt-PCR) analysis of mRNA is a procedure which can illustrate gene expression in all conditions using its complementary DNA (cDNA) as a template instead of the main mRNA molecule which represents the cellular transcription profile [19,20].

Real time PCR: The resulted cDNA samples covered a variety of mRNA sequence features which used for Real time-PCR experiments. PCRs were carried out according to the following profile: Real-time PCR assay conditions. Real-time PCR amplifications were conducted in a total reaction volume of 15µl, and comprised 5µl of 2X Premix Ex Taq (Perfect Real Time) reagents (TaKaRa), 100nM of each primer and 20ng of the template cDNA from the SMF treated cells and control ones. PCR experiments were performedusing ABI 7500 apparatus (Applied Biosystems) according to the following set up: initial template denaturation at 95°C for 15s, followed by 40 cycles at (95°C for 15s, 60°C for 20 s and 72°C for 20s). The second derivative maximum algorithm was used to calculate the Cycle Threshold (CT) value. All runs included negative and positive controls and were tested in duplicated. T the quantity of cDNA, relative to a reference gene, was calculated using the formula $2^{-\Delta CT}$ where ΔCT is equal to CT sample cDNA - CT reference cDNA [21,22].

Equation 2.1

ΔΔCt = ΔΔCtsample -ΔΔCt calibrator (Fold change = 2 -ΔΔCt)

ΔΔCt for each sample was also measured according to the equation where calibrator means endogenous reference gene. Statistical analysis of real time

Equation 2.2

SD fold change = (ln2) (st.dev ΔΔCt) (2-ΔΔCt)

Data was expressed as means and Standard Deviations (SD), normalized and P value ≦ 0.05 considered to be the statistical significant. Quantitative expression for the genes was normalized and analyzed using real time PCR procedure and one-way ANOVA Tukey test [23]. Human *Nanong* and *KCNH*-1 primers were designed and human *GAPDH* was also selected for positive control in the PCR reactions and all primer sequences are shown in table1.

Results

We have proved that the cord-derived cells were mesenchymal stem cells because of the observed expression of HMSCs surface antigens and the absence of hematopoietic ones. The lake of HLA-DR surface antigen was also examined and reported in our previous study showed the immunosuppressive properties of MSCs. The extracted cells have also differentiation ability into bone, cartilage and fat. Cord-derived cells' triple differentiation ability was another evident in addition to surface antigen representation; therefore, the extracted cells are MSCs and can also align in a parallel lines manner due to the static magnetic field exposure More explanatory data were presented elsewhere [15].

In this study all experiments were performed in neural differentiation culture conditions. Some changes occurred after 8 hours of SMF exposure and MSCs were observed in an extended and self-aligned manner as well as is reported in our previous studies.

Figure 2 part1 indicates that HMSCs have grown in a tree-like natural pattern, while they are observed to be ordered in a parallel alignment after SMF exposure.

All samples were cultured in the differentiation medium and light microscope photographs showed that SMF exposure induced more morphological changes in the treated samples as well as cell alignments which are evident in figures 2 and 3.

Figure 3 shows SMF effect on cell morphology in samples which were chemically induced by RA and then cultured for 2 and 3 weeks, indeed all conditions were equal for control and treated samples except magnetic exposure.

Some oligodendrocyte-like features have been observed in the SMF exposed cells beyond two weeks post-exposure culture time. It is clear that the features are more complete after 3 weeks which is illustrated in figure 3: B and D. Next experiments were performed to reveal whether the treated samples pose some of the neural tissue gene expression models or not.

RT-PCR

RNA extracted from HMSCs which were cultured in the presence and/or absence of treatment with SMF were used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA.

Polymerase chain reaction was then performed for cDNA samples and PCR products were studied using agarose gel-electrophoresis method [19,20].

Enhanced expression of pluripotent markers; *Oct-4*, *Sox-2* and *Nanong* in stem cells is well stablished in the recent investigations [24]. It is clear that these genes expression decreases either by inducing differentiation or in advanced development stages.

Figures 4,5 and 6 represent the results after agarose gel-

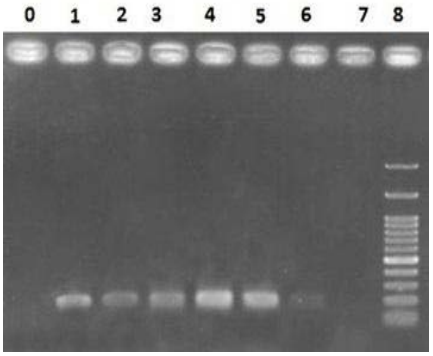

Figure 5: *Sox-2* PCR products agarose gel electrophoresis photograph; 1: SMF treated sample after 3 weeks; 2: RA treated sample after 3 weeks; 3: SMF and RA treated sample after 2 weeks; 4: human *GAPDH* (control+); 5: control sample (without SMF and RA) after 3 weeks; 6: SMF and RA treated sample after 3 weeks; 7: control- (without *Sox-2* primer) 8: Ladder.

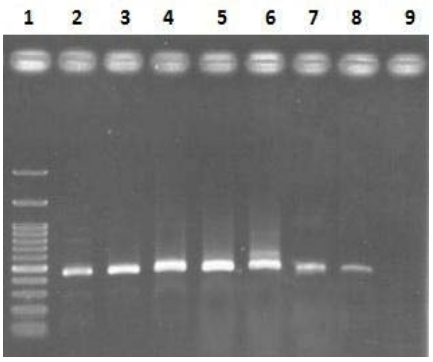

Figure 6: *Nanong* Rt-PCR products gel-electrophoresis; 1: Ladder 2: SMF treated sample after 1 weeks; 3: control non-exposed sample after 2 weeks; 4: control non-exposed sample after 3 weeks; 5: *GAPDH* (control+); 6: control sample after 3 weeks; 7: SMF treated sample after 2 weeks; 8: SMF treated sample after 3 weeks; 9: control- (without *Nanong* primer).

electrophoresis of the PCR products. The resulting amplified cDNA fragments are visible as the defined bands representing the defined genes expression.

RT-PCR experiment showed the mentioned genes expression increased in the control non-exposed samples but decreased in the treated ones as is evident in agarose gel-electrophoresis photographs (figures 4-6). *Oct-4* gene expression decline due to SMF exposure after three weeks post exposure culture time can be observed in Figure 4 number 4.

Figure 6 illustrates that *Nanong* expression was decreased after 18mT SMF exposure and culture for three weeks compared to the control non-exposed samples. Stemness markers including *Nanong* are highly expressed in stem cells while; differentiation causes these genes expression decline. Figure 6 shows that SMF exposure caused *Nanong* gene product decrease after culturing for two and three weeks (7 and 8 respectively). Lighter observed bands in the agarose gel for the SMF treated and three weeks cultured samples showed less Rt-PCR products amplification and decreased expression of the stemness genes indicating advanced differentiation.

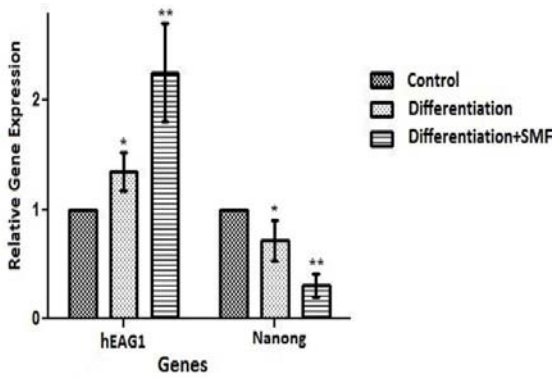

Figure 7: Real time-PCR data analysis revealed significant gene expression changes in the presence of neural differentiation culture medium and 18mT/8h SMF exposure. Control samples were cultured in the absence of SMF and differentiation medium.

Since *Nanong*, *Sox-2* and *Oct-4* genes are highly expressed in the differentiated cells [24], their decreased expression in RA and SMF treated cells after three weeks incubation shows that they are in the more differentiated state than the less cultured and/or control non-exposed samples.

Real timePCR

Our experimental groups include the following;

1. Control samples without SMF exposure and differentiation medium.

2. Chemically retinoic acid induced samples for neural differentiation.

3. SMF exposed chemically retinoic acid induced samples for neural differentiation.

In this experiment gene expression fold for chemically induced differentiation and both chemically and SMF treated samples were compared to the control non-exposed ones.

According to previous studies neural differentiation is accompanied by potassium channel genes expression including *EAG1* [25].

Human Ether a go-go1 gene (*hEAG-1*) also called KCNH1 or $K_v10.1$ encoding a protein which is a voltage-gated potassium channel and predominantly expressed in the Central Nervous System (CNS) and mutations leading to decreased *EAG1* protein level cause epilepsy, hypoplasia and multisystem developmental disorder characterized by intellectual disability. Therefore, hEAG-1 gene has been characterized as a neural differentiation marker [26,27].

Our data showed a significant decrease of the pluripotency (*Nanong*) gene expression in the presence of Retinoic Acid (RA) differentiation inducer (*: $P<0.05$) but SMF exposurecaused more cell differentiation (**: $P<0.01$).

This study showed that *hEAG1* gene expression significantly increased (**: $p<0.01$)after both treatments; RA chemical induction for neural differentiation and magnetic field exposure after three weeks culture time as illustrated in (Figure 7).

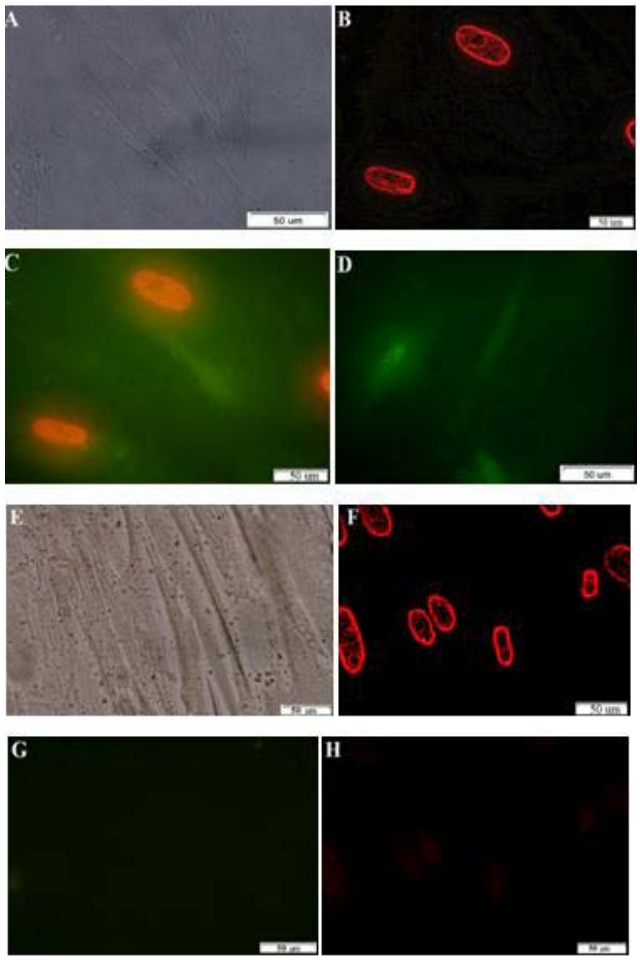

Figure 8: Immunocytochemistry experiment photographs: A: RA and SMF treated and cultured in neural differentiation medium samples light microscope photograph; B: the same sample PI stained fluorescence microscope photograph, cell nuclei absorbing PI and are observed in red; C: the same sample after ICC treatment for Nestin protein presence assessment which merged to B; D: Green colored filaments indicating cytoplasmic Nestin protein in the treated sample; E: RA treated sample without SMF exposure (control sample) light microscope photograph; F: the same sample PI stained fluorescence microscope photograph cell nuclei are seen in red; G: after ICC treatment for Nestin protein presence, no cytoplasmic Nestin protein is observed; H: merged picture of F and G.

Immunocytochemistry (ICC)

Nestin, a cytoplasmic protein found in neural stem cells is most highly expressed by differentiation into neuronal tissues namely oligodendrocytes [28]. Our results were in agreement with the previous findings and our real time-PCR results indicating neural differentiation. Nestin protein has significantly been increased in the RA treated samples after 8 hours of 18mT SMF exposure and three weeks culture in neural cell favored medium (Figure 8).

In this experiment samples were divided to two groups; 1- Retinoic acid induced and cultured in the neural tissue fevered medium for three weeks as control samples, 2- The identical samples which were exposed to SMF for 8 hours as treated ones.

After light microscope observation, the samples were stained by Propidium Iodide (PI) which binds to DNA and is used to illustrate DNA containing organelles just like Nuclei using fluorescence microscope.

Nestin antibody stained samples were then assessed by fluorescence microscopy and the photographs showed that the samples with both SMF and RA treatments posed significantly more Nestin protein expression which is one of the neural-like cell lineages characteristics [29].

PI and Nestin antibody stained photographs were then merged and the results are evident in the figure 8. Cytoplasmic Nestin protein green filaments are significantly observed in the SMF treated samples while it is less expressed in the control non-exposed samples (Figure 8).

Statistical analysis

Relative gene transcription data calculations carried out using Real Time quantitative -2 ΔΔCTmethod [19] (Livak & Schmittgen, 2001). Data analysis was performed to compare the fold of gene expression and determine significant changes using Graph Pad (Prism5) Software Analysis of Variance (one-way ANOVA, Tukey's test). P values were compared for all experimental groups and $p<0.05$ considered as significant and refuse null hypothesis [23].

Discussion

This review has discussed a number of already stablished stem cell studies and new strategies in the field of SMF differentiation effects and some of their potential uses for regenerative medicine research. Stem cells are present in all human organisms and capable of reproducing themselves or switching to become more specialized cells in human body such as cells in brain, heart, muscles and kidney [1].

Recent advancements in basic and clinical research on bone marrow, embryonic, fetal, amniotic, umbilical cord, and adult stem cells have revealed multiple possibilities for stem cell potential therapeutic applications which emerge as new powerful tools for tissue engineering and regenerative medicine [2].

Human Mesenchymal Stem Cells (HMSCs) that represent a mesoderm-derived population of progenitors, can easily be expanded *in vitro* and are also capable to differentiate and create cell lineages which exhibit the potential to repair or regenerate damaged tissues [30,31].

Other results confirmed that SMF treated cell cultures posed less proliferation rate which were in agreement with other investigations in this field for instance the moderate intensity SMF exposure effect on cell fate [7].

These cells have recently been one of the main subjects of regenerative medicine research and the large interest in MSC applicability for clinical approaches relies on the ease of their isolation from prenatal tissues, immunosuppressive and differentiation properties most notably for diseases related to the organs which have poor regenerative capability just like neural tissues [32].

However, bone marrow is characterized as a main source of MSCs, the method is considered to be painful and invasive and many scientists prefer to obtain MCSs from the other resources. Umbilical cord has been known as a proper reservoir for Human Mesenchymal

Table 1: Primer sequences.

Rt-PCR	
Sox2	Forward: TGGACAGTTACGCGCACAT
	Reverse: CGAGTAGGACATGCTGTAGGT
Nanong	Forward: ACCTATGCCTGTGATTTGTGG
	Revers: GAGTAGAGGCTGGGGTAGG
Oct4	Forward: CTTCTC GCC CCC TCCAGG T
	Reverse: AAATAG AAC CCCCAG GGTGAG C
Real time PCR	
Nanong	Forward: ACCTATGCCTGTGATTTGTGG
	Reverse: AAGAGTAGAGGCTGGGGTAGG
hEAG1	Forward: GGCCTTGACCTACTGTGATCTGCReverse:TCCGGAACACAATCCTCTTCCTC
GAPDH	Forward: GACCTCAACTACATGGTTTAC; Reverse: CTCCTGGAAGATGGTGATG

Stem Cells (HMSCs) which are promising candidates for development of future strategies in regenerative medicine. Umbilical cord-derived HCMSCs have been chosen for this study because they do not entangle the researcher in ethical issues associated with harvesting stem cells from live donors [33].

Many cells in a living body can divide and regenerate themselves but human embryonic neural tissue stop dividing in the first fetal developmental stages because the whole nervous system is based on inter-neuronal connections and adding an extra cell would mess up these connections and alter both the functionality and stored information in the central nervous system so human nerves stop dividing in the early stages of fetal development and diseases related to them cannot be normally cured by body natural healing power [34].This shows why human neurological disorders such as Multiple Sclerosis (MS), Parkinson's disease, and spinal cord injury which are related to loss of neurons and Glial cells in the brain or spinal cord are rarely been cured.

Stem cell-based therapy has provided new clinical strategies in this area. Although stem cells and their progeny can easily proliferate, make large populations and replace the lost tissues because of their self-renewal ability, the risk profile of stem cell therapy depends on many factors including the type of stem cells, their differentiation status and proliferation capacity *in vitro*, etcetera [35].

Scientists on observing cancer tissue under the microscope in the mid-19th century, noticed the similarity between embryonic and cancer tissues and suggested that tumors arise from embryo-like cells. This relationship among the nature of normal stem cells (embryonal, germinal and somatic) to cancer cells is now stablished. Cell signaling pathways shared by embryonic cells and cancer cells suggest a possible link between embryonic and malignant cells. Cancers result from continued proliferation and fail to differentiate and die in the proliferative zone; therefore, stem cells before grafts must be differentiated to help living body and prevent tumor formation [2,36,37]. Signals that control normal cell development may eventually lead us to insights in treating cancer by inducing tissue differentiation in a procedure called differentiation therapy [37,38]. Retinoid Acid (RA) is one of the chemicals which has been used in this approach [8], so was used as a chemical inducer in this study because of its ability to prevent cancer via differentiation enhancement has highlighted its role in the both research and clinical appliances [39].

Indeed, primitive cells (stem cells and their progenitors) are more sensitive to the environmental changes including magnetic field exposure than the other body tissues and capable to represent low energy field effects including SMF differentiation impacts [40].

Some of research projects have shown the effect of magnetic fields on the morphology and differentiation of certain stem cell types has recently been reported. Pacini et al. studied static magnetic field impact on human neural progenitor cell cultures and demonstrated that significant effects on human neuronal cells. The data was consistent with induction of differentiation without genome instability [41].

Several researches were performed to investigate how SMFs influence living systems. One of the speculated mechanisms of magnetic fields effect was cellular free radical accumulation which is considered as a well-known mechanism to describe *invivo* and *invitro* SMF interactions. According to the previous studies carried out in our laboratory, SMF exposure cause increased activity of Superoxide Dismutase (SOD) and catalase and ascorbate peroxidase decreased activity which in turn result in free radical accumulation [42].

Reactive oxygen species or ROS (oxygen radicals $O^{\cdot}$ and $O2^{\cdot}$) which are biological free radical carriers are used as some messengers in normal cell functions but in oxidative stress, high ROS levels may disrupt normal physiological pathways and result in differentiation induction [43]. As moderate intensity SMF effect of differentiation induction is stablished in other studies [44], it can be speculated that ROS accumulation due to the exposure may contribute cell fate determination.

Stemness factors just like *Sox-2*, *Nanong* and *Oct-4* expression (which have been known as undifferentiated and pluripotent cells characteristics [24]) were reduced after SMF exposure indicating differentiation process promotion.

Stem cells have differentiation capability [45] and Retinoic Acid (RA) can prevent cancers by differentiation induction in the proliferative cells [39,46,47]. Retinoids have also been investigated to cause neural differentiation in stem cells and progenitors [48,49].

Surface antigen representation in the cord-derived cells in addition to the ability of cord-derived cells to differentiate into osteocytes, chondrocytes and adipocytes proved that the cord-extracted cells are MSCs. Furthermore, SMF can induce differentiation and cellular alignment in cord-derived HMSCs without genome instability as we reported in our previous study [15]. In this investigation the treated samples were induced with RA which was excluded right after SMF exposure and at the same time for the non-exposed corresponded control ones. All samples were then cultured in neural differentiation fevered medium. After three weeks all cells were harvested and prepared for next experiment steps to find out whether SMF exposure can improve HMSCs differentiation into neural-like progenitor cells or not.

The SMF-induced morphological changes which exhibited branched dendrite-like features were more complete after three weeks in the SMF treated samples so we speculate that culture time is necessary for the differentiation process development as illustrated in figure 3; B&D. Rt-PCR agarose gel photographs also showed the decreased expression of *Sox-2*, *Nanong* and *Oct-4* genes after and 8 hours of 18mT SMF exposure which have been in progress for three weeks post-exposure culture time (figures 4,5 and 6). Consequently, least three weeks culture time after the treatment is needed to observe some of neural differentiation characteristics.

Furthermore, significant increase in *hEAG1*and Nestin genes expression which were identified as some of neural cell characteristics [27] as well as *Nanong* decreased transcription suggest neural differentiation promotion due to RA and SMF treatments and three weeks culture in neural fevered medium (Figures 7&8).

This approach may be considered as a way for providing precious progenitor cells for therapeutic applications and can create a paradigm for future studies to give insights on the usage of stem cells as potential tools for regenerative medicine.

Conclusion

As neural cell defects have very limited intrinsic healing capacity, development of new treatment methods for nervous system injury is of major importance for neurologists. Umbilical cord appears to be an ideal candidate to serve as a HMSCs' reservoir. Physical inducers just like SMF in moderate intensities along with the chemical ones (RA) can enhance HMSCs neural differentiation to produce neural-like cell lineages and this in turn can possibly facilitate cell-based neural disorders treatment. However further experiments should be performed to strongly address this point.

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