Morphological changes induced in Avian Nucleated Blood Cells by Extremely low Frequency Electric Fields

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Morphological changes induced by Extremely Low Frequency Electric Fields

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Morphological changes induced by extremely low Frequency Electric Fields

Abstract – In this paper, morphological effects of electric fields on Avian Erythrocytes (nucleated red blood cells) have been studied in detail. Morphological changes include rounding and cytoplasm transparency. It has been shown that the effect is non-thermal. Careful imaging and image analyses have been carried out to show that the degree of this effect is frequency dependent, and has a higher conversion rate at higher temperatures. Furthermore, to better understand the mechanisms behind the morphological changes, we investigated the dedifferentiation hypothesis and performed a series of tests on Avian Erythrocytes including fluorescence spectroscopy for hemoglobin, and tests on Human Umbilical Cord Blood, Mesenchymal Stem Cells, and Bone Marrow Mesenchymal Stem Cells including flow-cytometry analysis for expression of certain markers and calcium staining.

Keywords: cell morphology; extremely low frequency; electric field; non-thermal effects; dedifferentiation
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Introduction

Electric fields (EF) affect growth, regeneration, survival rate, organization, and activity of cells in-vitro [Bassett, 1993; McLeod et al., 1992; Pilla, 1974; Stuchly and Dawson, 2000]. According to one hypothesis, endogenous EFs can enhance wound and bone repair through a mechanism of signaling that results in dedifferentiation of cells in the fracture [Becker, 1961; Becker and Murray, 1967]. It has been observed in numerous studies that externally produced EF can be used as a tool for the reconstruction of bone tissue [Andrew et al., 1974; Fitzsimmons et al., 1992; Luben et al., 1982; McLEOD and Rubin, 1992] and possible improvement of non-union fractures. EF has been used for wound repair and regeneration of lost tissues [Alvarez et al., 1983; Bogie et al., 2000; Reger et al., 1999; Suh et al., 2009; Ud-Din and Bayat, 2014]. Stimulation by EF also has a positive effect on neuronal regeneration [Baptista et al., 2008; Francis et al., 2003]. Researchers have also observed the ability of regeneration and complete reconstruction of lost tissues in mammals [Becker and Spadaro, 1972]. At a cellular level, it has been observed that these fields are effective in growth and proliferation of cells in-vitro [Fitzsimmons et al., 1989; Jaffé and Nuccitelli, 1977; Sisken and Smith, 1975] and also in metabolism, and cell activity levels [Berg, 1993].

The regeneration of damaged organs of the body is observed naturally in the amphibians, such as a severed tail. It has been observed in these organisms, that almost immediately after limb amputation, blastema cells are present in the wound region and these cells contain undifferentiated cells [Alibardi, 2018]. There is evidence to suggest that the appearance of blastema cells depends at least partly on the process of dedifferentiation [Neff, 2018]. In one study, a salamander's hand was cut off and the arm bone was removed, in this situation, there is no bone or cartilage cell to migrate and produce similar cells. Nevertheless, it was observed that the hand of the salamander was regenerated as it was before, which suggests that bone and cartilage cells have been regenerated from muscle cells that have been
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dedifferentiated [Sandoval-Guzmán et al., 2014; Thornton, 1938; Wang and Simon, 2016], probably due to the endogenous EF in the body. In a study of mammalian bone marrow, a flow of about 5-15 nA could accelerate blastema formation in the bone fracture site and improve the recovery of injury with a faster process [Becker, 1972]. The results of these studies support the hypothesis that "the application of certain EF to cells can initiate dedifferentiation". The clinical importance of this topic is obvious when one considers the recent trends in cell-therapy.

It has also been observed that the use of Direct Current (DC) EF, as well as Alternating Current (AC) fields, can cause morphological changes towards stem-like cells in amphibian's Nucleated Red Blood Cells (NRBCs) [Pilla and Margules, 1977]. The change in the concentration of ions (such as calcium) has been suggested to play an important role in these transformations [Pilla, 1974]. In a work by [Smith et al., 1978], the effects of 50 Hz EF with an amplitude of 100 mV p-p (peak-to-peak) was investigated. A special applicator was constructed using tantalum wire, and the cells were exposed to EF for 1 h and the results were evaluated for up to 4 h after the application of the field. It was observed that the cells lose their spindle shape and became more spherical and there were light radii around their nucleus. When a calcium blocker (lanthanum chloride) was used, the cells did not show any change. This supports the role of calcium in morphological changes, and possibly in dedifferentiation.

In the present paper, we have re-examined the morphological effects of extremely low-frequency EF on avian erythrocytes with several additional studies. Remarkable morphological changes have been observed. Following EF treatment, cells were carefully observed for changes in morphology such as cells shape changing from spindle-shaped to spherical and cytoplasm color change related to the last stage of morphological transition. Several frequencies in the range of 5-50 Hz were studied to understand the frequency dependence of these changes, together with statistical analysis of different frequencies. The effect is shown to be frequency-dependent. A complete video has been made of the nearly perfect transition of
all cells over a time span of three days at room temperature. Since the morphological changes in avian erythrocytes are suggestive of possible dedifferentiation effects, we hypothesized that the reason for the change in color can be the loss of hemoglobin. There is high hemoglobin content in erythrocytes and the loss of this hemoglobin can suggest that cells are undergoing a dedifferentiation process. In this paper, we have tested this hypothesis using fluorescence spectroscopy techniques.

Further additional tests were carried out for cells with known surface proteins to understand the relationship between the applied fields (the same amplitude and frequency) and transition of cells into less-differentiated states. We tested human umbilical cord nucleated blood cells (UCB-MSCs) and human bone derived mesenchymal stem cells (BM-MSCs) for indications of dedifferentiation using flow cytometry and marker analysis. In this test, UCB-MSCs samples were examined using cell markers CD34, CD71, which are specific markers for human blood cells. After the application of the field, an increase in the expression of CD34 and reduction in the CD71 were observed. These changes suggest a transition of cells towards less differentiated states.

Materials and methods

Exposure system

An EF exposure system was fabricated by placing two Tantalum electrodes (handmade) in a 6 cm diameter polystyrene petri dish (Sigma Aldrich, St. Louis, MO). Tantalum is a component that is highly compatible with biological environments and cell culture media [Leng et al., 2006; Pilla and Margules, 1977]. One electrode is located at the center of the petri dish, formed by a vertical single wire extending to the bottom of the dish. The other electrode is at the periphery of the container, and is made by wrapping 32 turns of tantalum
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wire around a 0.5 cm diameter silicone hose (Fig. 1). The distance between the two electrodes is 2 cm and 0.5 mm diameter tantalum wire is used.

The field is applied using a laboratory function generator (LH Funktionsgenerator 2 MHz, 725 501, Germany). The voltage waveform applied to the apparatus is a zero-centered square pulse with an amplitude of 200 mV p-p, and applied frequencies of 5, 15, 25, and 50 Hz in separate culture dishes.

Dosimetry

Field simulation is performed using a commercial simulator software CST (Computer Simulation Technology, Dassault Systemes, Germany), used to assess EF values within the culture dish. We note that using simulated field values is current practice because measurement of EF values in bioelectromagnetics is often difficult without perturbing the actual experiment.

In this simulation, electrode-electrolyte interface properties such as electrical conductivity and relative permittivity were calculated using mathematical descriptions performed by Richardot and McAdams [2002] [Richardot and McAdams, 2002]. Electrical conductivity value was $5 \times 10^{-5}$ S/m and relative permittivity was $7.2e7$ F/m. Also, electrodes are tantalum metal with $0.025$ cm radius for the central electrode and $2$ cm inner radius and $2.5$ cm outer radius for the peripheral electrode. The thickness of the electrode-electrolyte interface model on the surface of the electrodes was $0.01$ cm. Petri dish used in this simulation had a $3$ cm radius and $1$ cm height. The treatment apparatuses designed in CST can be seen in the figure (Fig. 2). The simulation was performed for 200 mV p-p EF with a frequency of 50 Hz (Fig. 2).

The intensity of the electrical field near the central electrode was about $2.103$ V/m, near the peripheral electrode $0.055$ V/m and in the midway between the two electrodes $0.404$ V/m.
Cell cultures and maintenance

The trial was approved by the local investigation committee and conducted in accordance with the Declaration of Helsinki as approved by the identifier IR.ACECR.IBCRC.REC.1397.015.

Avian Erythrocytes

- 2 ml blood samples were taken from the subcutaneous vein of a female Columba Livia Domestica.

- The blood was diluted from one to two Hanks balanced salt solution (HBSS), (Sigma Aldrich).

- In a calibrated test tube, about 3 ml of the Ficoll (Sigma Aldrich) was poured, and the blood was slowly poured into the tube through the Pasteur pipette to avoid mixing with Ficoll until the total volume was about 7 cc. Then the tube was treated for 28 min with 2800 rmp centrifuge. The tube was then taken out of the centrifuge. Five different layers were formed inside the tube, which was from the bottom to top: RBC layer (heaviest layer), thin granulocyte layer, Ficoll layer, cell mononuclear (including lymphocytes and monocytes) seen in the form of a white and thin ring, and Serum layer (top layer).

The red blood cells were then counted and seeded at 1 million cells per petri dish with RPMI 10% FBS (Sigma Aldrich).

Umbilical Cord Blood Mesenchymal Stem Cells (UCB-MSCs)

- Immediately after removal of the cord of human infant, 10 ml blood samples from umbilical arteries from the clamping region of the umbilical cord was collected in a heparinized tube and examined for complete cell counting.

- The blood was diluted from one to two HBSS. Saline can be used instead of Hanks.
In a graduated test tube, about 3 ml of Ficoll was poured, and the blood was poured slowly with a Pasteur pipette into the tube to avoid mixing Ficoll until the total volume was about 7 cc. Then the tubes were treated for 28 min with 2800 rpm centrifuge. After that, the tube was taken slowly out of the centrifuge. Five different layers were formed inside the tube, which was from bottom to top: RBC layer (heaviest layer), Thin granulocyte layer, Ficoll layer, mononuclear cells (including lymphocytes and monocytes) seen in the form of a white and thin ring, and serum layer (top layer). The red blood cells were counted and seeded about 1 million cells per Petri dish with RPMI 10% FBS.

Bone Marrow Mesenchymal Stem Cells (BM-MSCs)

To differentiate BM-MSCs into osteocyte, MSCs (2 x 10^4 cells/cm^2) were incubated in CM until a confluent layer was achieved and then osteogenic medium was added, containing DMEM supplemented with 10 % FBS, 9 % HS (Sigma Aldrich), 2 mM L-glutamine (Sigma Aldrich), 100 U/mL penicillin (Sigma Aldrich), 100 μg/mL streptomycin (Sigma Aldrich), 50 ng/mL L-thyroxine (Sigma Aldrich), 20 mM β-glycerol phosphate (Sigma Aldrich), 100 nM dexamethansone (Sigma Aldrich) and 50 μM ascorbic acid (Sigma Aldrich). Medium was changed every 3-4 d. After 21 d, cells were fixed in 4 % formaldehyde and stained with 10 % Alizarin Red (Sigma Aldrich).

**Exposure protocol**

For avian erythrocytes and the test at 4 °C, after separation of cells, they were transferred to petri dishes. There were two controls, simple control and control-wire (sham) (control with Tantalum wire inserted but no voltage applied) and 4 treatments. Petri dishes were placed at room temperature and then, using the function generator 5, 15, 25 and 50 Hz fields were
applied to the treatment petri dishes for 1 h at each frequency. After each treatment frequency cells were transferred to a flask after changing the culture medium and subsequently to a 4 °C refrigerator and for the new test the treatment petri-dish was filled with new cells. Control petri-dishes were placed in the test environment for the whole experiment and then transferred to the refrigerator. The sham cells were transferred to a flask and placed in the refrigerator after 1 h, as with the treated samples.

For avian cells used in the filming process, the cells were kept at room temperature directly after exposure and time-lapse movie was made for three consecutive days. The same was done for the control movie.

For human UCB-MSCs and BM-MSCs, the treatment was performed for 50 Hz frequency. Afterwards, the cells were incubated at 37 °C with 5 % CO₂, followed by marker analysis.

**Experimental Analysis**

**Image and video capture**

The microscope used is a laboratory inverted biological microscope (XSD-4B, Chongqing MIC Technology, Chongqing, China). For pictures shown in this article, a magnification of 400x is used. For statistical calculations, pictures were taken by a magnification of 200x. Before and after the field exposure, then every day for four days. Each time, an average of 10 photos were taken from the corners and center of the flask (Fig. 3). These photos were used for optical investigation and statistical analysis.

**Statistical Analyses**

The significance of changes as evidenced by difference from control or difference between
days were statistically tested using the two-sample T-test on an Excel spreadsheet with a significance level of $P=0.05$. Significant differences in the average cell-counts are marked by (*) in Figure 5. Also, one-way ANOVA has been carried out on change percentages in images (as measurement variable) between frequencies (as nominal variable) to determine frequency dependence of the effects with a significance level of $P<0.05$.

**Fluorescence Spectroscopy**

Spectroscopy was performed by a Varian Cary Eclipse Fluorescence Spectrophotometer (*Agilent, Santa Clara, California*) at 295 nm and 405 nm wavelengths.

**Temperature and pH**

The temperature was measured by pocket thermometer (*IP65 Pocket Temp, HLP controls, South Windsor NSW, Australia*) and pH was measured by multi-parameter analyzer pH meter (*C561, Consort, Turnhout, Belgium*). Both parameters were observed before and after exposure.

**Flow cytometry**

Cell surface antigen expression of MSCs was performed at passage 2 (P2). Briefly, single cell suspension of stromal cells from all sources was prepared and washed twice with PBS and resuspended in PBS containing 5% FBS. Cell concentration for FACS was $10^6$ cells/mL. Fluorescein phycoerythrin (PE) and fluorescein isothiocyanate (FITC) conjugated anti-human antibodies were administrated for labelling following cell-surface epitopes: CD73-PE, CD90-FITC, CD44-FITC, CD105-FITC and CD73-FITC. After adding antibodies, the cell suspension was incubated at 4°C in dark for 30 min. The isotype specific anti-serum conjugated with FITC or PE were utilized as negative controls. After cell washing 10,000 non-
gated labelled MSCs were acquired and analyzed using a BD FACS Calibur instrument (BD Bioscience, Cambridge, United Kingdom). FACS data analysis was done using FlowJo software version 10.0.7 (Tree Star, Ashland, OR).

Staining:

BM-MSCs differentiated to osteocyte were stained by Alizarin Red for calcium concentration. The medium was removed and cells were thoroughly washed with PBS two times and then fixed with 4 % formaldehyde solution for 30 min. Following fixation, cells were washed with distilled water and then stained with 10 % Alizarin Red solution for 5 min. In the end, cells were thoroughly washed with distilled water and visualized utilizing a light microscope. Also, avian erythrocytes were stained with Trypan Blue (Sigma Aldrich).

Results

Avian Erythrocytes

Morphological changes:

Cells were photographed to detect the transformation of avian erythrocytes using optical microscopy. The images taken from transformed cells can be seen in Figure 4 (a). The cell images are arranged from the most differentiated state to the most embryonic-like state (Fig. 4 b). Deformation of cells from ellipsoidal to Spherical and then the loss of color, most probably due to hemoglobin loss is seen in Figure 4 (a) and (b). To be certain that these changes are not an indication of cell death, Trypan Blue staining results are also obtained for cell viability indicating living cells (Fig. 4 c). Radial streaks can be seen around the nucleus of cells after field exposure and before morphological changes begin, which can indicate the presence of high levels of RNA in these areas (Fig. 4 d) [Smith et al., 1978].
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Image and statistical analysis

Morphological responses to each frequency were determined by sequential imaging and counting the number of transformed cells in different containers corresponding to different frequencies. Careful eye-examination was carried out on the images to provide highly precise statistics. The cells were counted once every day for 4 days by capturing images as described in image and video capture section. Blood cells were counted and examined by careful eye-examination. The results are provided in Figure 5. In these graphs, the average number of transformed cells in the images received from each container has been determined, there is a significant difference between the treatment and control cells, especially at 50 Hz (Fig. 5).

Numerical values for total transformed cells are also given in the table (Table 1). One-way ANOVA between different treatment frequencies on the fourth day supports significant ($P = 2 \times 10^{-7}$) difference in change percentages (calculated as changed cells per total), indicating that the effect is frequency-dependent. (Table 2)

Also, changes in the total number of cells in captured images are indicated in Figure 6. According to this chart, the total number of cells in the control and sham, as well as the frequency of 25 Hz, is always reductive, but in other cases, the cell death is much delayed (Fig. 6 a). We note that the number of total cells is estimated only approximately based on photographs, but the general behavior that is observed can be interpreted as increased viability of transformed cells. This is supported by the fact that 25 Hz, The frequency with low viability, is the same frequency at which nearly no transformation occurs (Fig. 6 a). The exact number of these countings are mentioned in Table 3. In Figure 6 (b), the ratio of changed cells per total cells at frequencies of 15 Hz and 50 Hz has been illustrated. As can be seen, the ratio of the transformed cells to the total has an increasing trend with time for both frequencies.
The statistical results in Tables 1, 2 and 3, are obtained from cell samples that were kept at 4 °C after treatment, where the conversion rate of cell morphologies was lower. In another round of experiments, we kept the cells at room temperature after treatment. This greatly accelerated the morphological transformation and nearly all cells underwent transition in three days. This morphological transition at room temperature has been captured by sequential image acquisition from the cells using an inverted microscope. From the beginning of the field exposure to 3 days after, video frames are taken every 60 seconds. These results are available in the attached video (Video 1) for treatment and (Video 2) for control. A series of snapshots from the video showing cells in different states of conversion can be seen in Figure 7.

Morphological changes were also observed in a separate test carried out on avian erythrocytes in their native blood serum i.e. no separation process before field exposure (Fig. 8). However, quantitative analysis similar to those for separated cells turns out to be much more difficult here due to the high number of other proliferating cell-types present, because some of these cells share several geometrical similarities with our altered erythrocytes. Alterations are also observed in the control-wire sample.

Florescent spectroscopy:

A fluorescence spectroscopy test was used on cell culture media to understand if the color change of the cells is due to losing their hemoglobin. The spectroscopy results for 405nm and 295nm excitation wavelengths are presented (Fig.9). These two excitation wavelengths are chosen to excite the 316-370 nm resonance and 440 nm resonance peaks of hemoglobin [Yang et al., 1998; Zheng et al., 2011] As it can be seen in Figure 9, the treatment cells have a lower fluorescence intensity in comparison with control cells, more so for the 440 nm peak.
This is an expected result if EF treatment should cause intra-cellular hemoglobin to be added to the culture medium. This is because, for molarities in excess of several ten mM, concentration quenching leads to the lower intensity with increased concentration [Yang et al., 1998].

Temperature and pH:

Changes in temperature and pH were measured in both treatment and control cell cultures to test if the transformations are a result of pH and/or temperature change. The temperature of the room was 26.5 °C, the temperature of the treatment medium was 23.6 °C which was measured after applying field and the temperature reading of the control medium after application of the field was 23.3 °C. Also, no difference was observed in the pH of the two environments.

UCB-MSCs

As a complementary assay and to evaluate the hypothesis of dedifferentiation, human blood markers CD34 and CD71 were tested on EF-treated human UCB-MSCs (Fig. 10). The percentage of conversion on the first day for treated cells for the CD34 marker was 5.28 % (Fig. 10 e), as compared with 3.41 % for control and sham cells (Fig. 10 c). Also, the level of CD71 marker for control cells is 34.3 % (Fig. 10 b), while it is 33.2 % for treated cells (Fig. 10 f).

BM-MSCs

To evaluate the effect of EF on BM-MSCs, four markers were examined. These markers are CD44 and CD73 (Fig. 11), and CD90 and CD105 (Fig. 12). As can be observed in (Table. 4), before any EF treatment the level of markers expression prior to differentiation (Table. 4, Before Dedifferentiation) into bone marrow cells is low in relative to that after differentiation.
especially in sham cells (Table. 4, After Dedifferentiation – Control-Wire). After chemically induced differentiation into bone cells, these levels increase (Table. 4, After Dedifferentiation – Control-Wire). Following the EF-treatment of differentiated cells for 1 h, a decrease is observed (Table. 4, After Dedifferentiation – Treatment) indicating steps towards dedifferentiation. In other words, undifferentiated BM-MSCs have lower levels of markers expression that differentiated cells, and we observe that after EF treatment of differentiated cells, marker levels decreases significantly especially for CD44, CD73 and CD105 markers.

Staining:

The concentration of calcium in BM-MSCs was investigated using Alizarin Red staining to investigate the return of these cells to a less differentiated state. The results are imaged in Figure 13. Since we have used mesenchymal stem cells that were differentiated into bone cells which have high calcium concentrations, a decrease in calcium sediment indicates movement towards a less differentiated state, as confirmed by Alizarin Red staining (Fig. 13).

Dosimetry and CST:

The maximum possible change in culture medium temperature during EF application can be obtained by calculating the SAR (Specific Absorption Rate). Given the values 

\[ E_{\text{max}} = 2.103 \frac{V}{m} \] (from Figure 2), \( \sigma = 1 \frac{S}{m} \), and \( \rho = 1000 \frac{kg}{m^3} \) SAR can be calculated as \( SAR = \frac{\sigma}{(2 \rho)} = 0.0022 \frac{W}{kg} \). Considering an exposure time of \( \Delta t = 3600 \) s and an upper bound on a thermal capacity \( C = 4200 \frac{J}{kgK} \) the temperature change estimate will be \( \Delta T = \frac{(C \Delta t)}{SAR} = 0.0018 K \).

Discussion

It is instructive to see if the observed effects are thermal or non-thermal in nature. As shown in
the results section, the 0.002 degrees temperature change estimated by SAR calculation is an upper bound on temperature rise because no loss of heat due to convection or thermal conduction is assumed. Together with experimentally measured constant temperature results, this indicates that the morphological changes are not caused by temperature variation, i.e. the observed effects are non-thermal.

The culture medium was changed after each treatment of avian erythrocytes. This rules out the possibility that cell changes were caused indirectly by EF affecting the culture medium macromolecules. Also considering the negligible temperature variations, it can be concluded that the observed morphological changes are not due to energy transfer or changes in temperature and pH. The observations are thus discussed in a non-thermal context of EF effects on cells, and direct interaction between the EF and the cells are assumed. In many studies on dosimetry, the amount of energy transferred to cells is quite low, and it is clear that the observed effects are not due to energy transfer phenomena that change chemical activity [Pilla and Kaufman, 1984]. In this sense, the effects are understood as information transfer to cells, through interaction with EF. One of the main compartments of the cell that receives EF with is the cell membrane and voltage gated channels, notably calcium channels[Galt et al., 1993]. Known field effects with calcium-related mechanisms include alterations in the cell's behavior (Apoptosis, differentiation, etc.) and appearance [Pall, 2013].

In our study, the morphological changes observed were frequency-dependent and 25 Hz provided the lowest rate and 50 Hz the highest as compared to other test frequencies. The 50 Hz was used in experiments that were carried out on human cells as well as additional experiments on avian erythrocytes that aimed to elucidate the nature of these changes.

The morphological changes might be suggestive of dedifferentiation because transformed cells have apparent features that are mostly expressed by less differentiated cells.
These include smaller cytoplasm relative to the entire cell and large nucleus, a primary appearance with fewer organelles, and after all, spherical shape [Cai et al., 2007].

Surface markers for avian erythrocytes were not available, but markers were available for UCB-MSCs as well as BM-MSCs. CD71 and CD34 markers for UCB-MSCs can be related to the level of differentiation of cells. An increment in CD34 marker, and/or a reduction in CD71 marker, both indicate an increase in the level of stemness in target cells, which were observed as indicated in Figure 10. Also, CD44, CD73, CD90, and CD105 markers were examined for BM-MSCs. A higher level of these markers indicates cell differentiation. As can be seen in Table 4, the levels of markers before differentiation of the cells into the bone cells were low, and after differentiation, the expression of these markers increased. After EF application, the expression level was reduced, which supports dedifferentiation.

The color change in avian erythrocytes can be a further clue (Fig. 4). It is hypothesized that the reason for this change is due to a loss of hemoglobin. Spectroscopy was performed to investigate this hypothesis. We experimentally compared the hemoglobin content of the culture medium in control and treatment samples. The control and treatment media were tested for 295 nm and 405 nm excitation wavelengths. As shown in Figure 9, the treatment media revealed lower intensity for both wavelengths, especially for 438 nm. Considering the concentration quenching effect at high molarities, a lower intensity is expected for a higher concentration of hemoglobin. To see if the differences in Figure 9 are meaningful we estimate the molarity change assuming that all EF treated cells purged their hemoglobin content into the culture media (see supplement video). Hemoglobin proteins form approximately half of the erythrocyte volume so for each cell there is 150 \( \mu m^3 \) hemoglobin. Considering 87 \( nm^3 \) as the volume of one hemoglobin [Erickson, 2009] and \( 10^7 \) cells from our cell-count, we have a total of about \( 10^{16} \) hemoglobin proteins or \( 1.6 \times 10^{-8} \) mols inside all cells. Considering the fact that we have a 6 ml medium volume the molarity difference will be 2.5 \( \mu M \). With regard to the
relative sensitivity to changes in micro molsars in [Yang et al. 1998] the observed difference between control and treatment media (20 % for 438 nm and 10 % for 316-368 nm) can prove quite meaningful and we can conclude that our results for spectroscopy are significant especially for 438 nm wavelength, which is representative of hemoglobin.

In the comparative analysis of figure 5, the control/control-wire (sham) samples also show a degree of change. Although this is not significant relative to several applied frequencies, a possible explanation is electric induction from power-supply frequency magnetic fields that are present in the laboratory environment, and are coincident with the most effective treatment frequency of 50 Hz. It can thus be expected that these samples are exposed to weak amplitudes of 50 Hz.

Finally, in experiments performed on BM-MSCs, it was observed that, by applying the EF, the concentration of calcium ion for the treatment cells decreases as shown in Figure 13. This indicates the direct relation of the EF effects to calcium ion and its influence on stem-like changes. This is confirmed by research results reported by Pilla [1974] [Pilla, 1974], where calcium is one of the fundamental players in cellular activities. It has been shown that the use of calcium channel blocker prevents cell deformation [Smith et al., 1978]. These observations support our results for the role of calcium for BM-MSCs.

Considering naturally occurring dedifferentiation and the fact that the frequencies used in this work are routinely generated endogenously within the body (e.g. by the nervous system), dedifferentiation should not seem surprising. In some studies, human body’s self-induced dedifferentiation was investigated such as dedifferentiation of neuronal progenitors [Lin et al., 2017] and epidermal Gata6+ cells [Donati et al., 2017] both initiated by injury, which is closely related to electrical injury potentials and nerve signaling. Also, evidence of dedifferentiation in striated muscles has been presented [Frasch, 2016]. In another study dedifferentiation of astrocyte in central nervous system have been proven to be successful in
both in-vivo and in-vitro studies [Fu et al., 2018a] along with peripheral nerve [Fu et al., 2018f], musculoskeletal [Fu et al., 2018c], and skin [Fu et al., 2018e] regeneration caused by dedifferentiation. The possibility of dedifferentiation also was studied in human Adipocytes [Corsa and MacDougald, 2018; Fu et al., 2018b; Lessard et al., 2015] and in mice (in-vivo study). Dedifferentiation and its relation to regenerative medicine are well known today [Fu et al., 2018d; Pesaresi et al., 2018] and new tools to achieve regeneration without chemical or genetic manipulations can be quite promising.

**Conclusion**

The effects of several ELF frequencies on viable cells were investigated, and the dedifferentiation related morphological changes were carefully monitored and recorded during field application. The results suggest that morphological changes are related to calcium channel mechanisms and that they can be an indication of dedifferentiation. Marker analysis supports this conclusion for two types of human cells. Considering the medical impact of these findings, further research is needed to understand the underlying mechanisms.

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**Figure Captions**

Fig. 1. Treatment apparatus. (a) side view, (b) Top view. I: central tantalum wire electrode, II: The peripheral electrode (tantalum wire wrapped around silicone hose ring), III: petri dish.
top, IV: silicon hose ring, V: silicon holder, VI: bottom of the central electrode with circular sire, VII: peripheral electrode wire.

Fig. 2. Treatment apparatus and CST simulation. (a) With cell culture. (b) With electrode-electrolyte interface model layer. (c) Just electrodes. (d) Top view. (e) Side view.

Fig. 3. Flask imaging locations.

Fig. 4. Different morphological state of Avian Erythrocytes under effect of EF stimulation (a) Transformation of cells from mature (I) to most dedifferentiated (VII). Changing from spindle shape to spherical and loss of gray color can be observed. (b) Graphic depiction of the transformation process. (c) Trypan Blue staining. Brightness of stained living cells can be observed. (d) Streaks appear around the nucleus of changing cell before transformation.

Fig. 5. Average number of all morphologically transformed Avian Erythrocytes in captured images for each day. (a) First day. (b) Second day. (c) Third day. (d) Forth day. Error-bars show standard deviation and significant differences (P<0.05) are marked by (*). Trivially significant columns are not indicated for better clarity.

Fig. 6. Statistical demonstration of different groups of Avian Erythrocytes during EF treatment (a) Total number of cells in captured images. (b) Changed cells per total in 15 Hz and 50 Hz.

Fig. 7. Snapshots form Avian Erythrocytes under effect of EF stimulation video with different states of conversion. At the center: focused part of the images.

Fig. 8. Morphological changes observed in native blood serum (50 Hz)

Fig. 9. Spectroscopy results of Avian Erythrocytes after 50 Hz EF treatment. (a) 405 nm wavelengths. (b) 295 nm wavelengths.

Fig. 10. Flow cytometry charts for UCB-MSCs. (a) Control, CD34, 3.41 %. (b) Control, CD71, 34.3 %. (c) Control-Wire (sham), CD34, 3.41 %. (d) Control-Wire (sham), CD71, 32.3 %. (e) 50 Hz treatment, CD34, 5.28 %. (f) 50 Hz treatment, CD71, 33.32 %.
Fig. 11. Flow cytometry charts of CD44 and CD73 markers for BM-MSCs. (a) Control, CD44, 93.6 %. (b) Control, CD73, 93.5 %. (c) Control-Wire (sham), CD44, 96.2 %. (d) Control-Wire (sham), CD73, 95.6 %. (e) Treatment, CD44, 91.6 %. (f) Treatment, CD43, 89.8 %.

Fig. 12. Flow cytometry charts for CD90 and CD105 markers for BM-MSCs. (a) Control, CD90, 84.1 %. (b) Control, CD105, 84.3 %. (c) Control-Wire (sham), CD90, 90.8 %. (d) Control-Wire (sham), CD105, 94.2 %. (e) Treatment, CD90, 75.8 %. (f) Treatment, CD105, 86.8 %.

Fig. 13. Calcium concentration in BM-MSCs staining with Alizarin Red under effect of EF stimulation. (a) Before staining. (b) After staining, Control. (c) After staining, Control-Wire (sham). (d) After staining, Treatment. Scale bar represents 100 µm.

Table Captions

Table. 1. Sum of all transformed Avian Erythrocytes under effect of EF stimulation, numerical values.

Table. 2. Analysis of variance for frequency dependence of morphological effects.

Table. 3. Total number of Avian Erythrocytes, numerical values.

Table. 4. BM-MSCs CD44, CD73, CD99 and CD 105 markers before and after dedifferentiation under effect of 1 h EF treatment
Treatment apparatus. (a) side view, (b) Top view. I: central tantalum wire electrode, II: The peripheral electrode (tantalum wire wrapped around silicone hose ring), III: petri dish top, IV: silicon hose ring, V: silicon holder, VI: bottom of The central electrode with circular sire, VII: peripheral electrode wire.

209x283mm (300 x 300 DPI)
Treatment apparatus and CST simulation. (a) With cell culture. (b) With electrode-electrolyte interface model layer. (c) Just electrodes. (d) Top view. (e) Side view.

147x112mm (300 x 300 DPI)
Flask imaging locations.

146x60mm (300 x 300 DPI)
Different morphological states of Avian Erythrocytes under effect of EF stimulation (a) Transformation of cells from mature (I) to most dedifferentiated (VII). Changing from spindle shape Fig.4. to spherical and loss of gray color can be observed. (b) Graphic depiction of the transformation process. (c) Trypan blue staining. Brightness of stained living cells can be observed. (d) Streaks appear around the nucleus of changing cell before transformation.

212x293mm (299 x 299 DPI)
Average number of all morphologically transformed Avian Erythrocytes in captured images for each day. (a) First day. (b) Second day. (c) Third day. (d) Forth day. Error-bars show standard deviation and significant differences (P<0.05) are marked by (*). Trivially significant columns are not indicated for better clarity.
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Caption: Sum of all transformed Avian Erythrocytes under effect of EF stimulation, numerical values.
### Analysis of variance for frequency dependence of morphological effects

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<th>Sum of squares</th>
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<th>Mean square</th>
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Caption: **Analysis of variance for frequency dependence of morphological effects.**
Statistical demonstration of different groups of Avian Erythrocytes during EF treatment (a) Total number of cells in captured images. (b) Changed cells per total in 15 Hz and 50 Hz.
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Caption: Total number of **Avian Erythrocytes**, numerical values.
Fig. 7. Snapshots from Avian Erythrocytes under effect of EF stimulation video with different states of conversion. At the center: a focused section of the images.

1021x578mm (72 x 72 DPI)
Fig. 8. Morphological changes observed in native blood serum (50 Hz)

348x74mm (96 x 96 DPI)
Spectroscopy results of Avian Erythrocytes after 50 Hz EF treatment. (a) 405 nm wavelengths. (b) 295 nm wavelengths.
Flow cytometry charts for UCB-MSCs. (a) Control, CD34, 3.41 %. (b) Control, CD71, 34.3 %. (c) Control-Wire (sham), CD34, 3.41 %. (d) Control-Wire (sham), CD71, 32.3 %. (e) 50 Hz treatment, CD34, 5.28 %. (f) 50 Hz treatment, CD71, 33.32 %.

297x257mm (95 x 95 DPI)
Flow cytometry charts of CD44 and CD73 markers for BM-MSCs. (a) Control, CD44, 93.6 %. (b) Control, CD73, 93.5 %. (c) Control-Wire (sham), CD44, 96.2 %. (d) Control-Wire (sham), CD73, 95.6 %. (e) Treatment, CD44, 91.6 %. (f) Treatment, CD43, 89.8 %.

306x260mm (95 x 95 DPI)
Flow cytometry charts for CD90 and CD105 markers for BM-MSCs. (a) Control, CD90, 84.1 %. (b) Control, CD105, 84.3 %. (c) Control-Wire (sham), CD90, 90.8 %. (d) Control-Wire (sham), CD105, 94.2 %. (e) Treatment, CD90, 75.8 %. (f) Treatment, CD105, 86.8 %.

300x259mm (95 x 95 DPI)
Calcium concentration in BM-MSCs staining with Alizarin Red under effect of EF stimulation. (a) Before staining. (b) After staining, Control. (c) After staining, Control-Wire (sham). (d) After staining, Treatment. Scale bar represents 100 µm.

1460x1092mm (72 x 72 DPI)
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Caption: BM-MSCs CD44, CD73, CD99 and CD 105 markers before and after dedifferentiation under effect of 1h EF treatment.
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Date: 2019.03.14 Contributor name: Mehrdad Saviz (also on Behalf of all co-contributors)

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