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The Effect of Static Magnetic Field on *Paramecium caudatum* Under Calcium Channel Blockade with Magnesium Sulfate

Atefeh Babazadeh[1], Manizheh Karami[1, 2*]

1. Department of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran
2. Neurophysiology Research Center, Shahed University, Tehran, Iran

Abstract

Background and Objective: According to evidence, the effect of static magnetic field (SMF) against motor learning deficits has been attributed to the effect of the field on calcium (Ca) channel conductance, but it is not fully understood. We treated *Paramecium caudatum* with a Ca channel blocker, magnesium sulfate (MgSO4), in the presence or absence of SMF.

Materials and Methods: *P. caudatum* was collected from temporary ponds, specifically identified, and cultured on straw. After sequential passage, the pure medium was divided into two media and placed in standard laboratory conditions or SMF (72 hours). A small drop (0.1 mL) of each medium was placed on the slide and studied 25 times under x40 magnification during 30 seconds period with 5 seconds intervals. The control groups only received 1 µL of distilled water. Different doses of MgSO4 (1, 4, 8, 16 and 32 µg/µL) were used in both conditions. The swimming and avoidance behavior of the animal was recorded live, and after fixation, the pellicular motor system, Ca channel and macronucleus were carefully examined. All data were analyzed using ANOVA.

Results: The swimming movements of animals in the field were intensely reduced when treated with MgSO4. The use of the blocker also caused the destruction of some pellicular and macronucleus structures in the presence of SMF.

Conclusion: With the use of MgSO4, the adverse effects of SMF on the motility and structure of *Paramecium* were enhanced, indicating that Ca ions play an important role in the protective effects of the magnetic field.

Keywords: *Paramecium caudatum*, Static Magnetic Field, Swimming, Avoiding, Magnesium Sulfate

1. Introduction

Zoologists classify animals into two main sub-branches, unicellular and multicellular. Protozoa are eukaryotic and basically single-celled organisms, and despite the fact that they are considered the simplest animals, they have a very complex structure, because this complexity is entirely unique to one cell and all life actions must be carried out by a single cell. Therefore, no matter how complex its structure is, it is physiologically balanced (1). This animal can be grown in the laboratory with a little straw or hay and some of them such paramecia can be seen even with the naked eye. *Paramecium* usually has a size of 100-300 µm, and it is egg- or oval-shaped. The main features of this animal are cilia. This simple animal, which

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***Corresponding Author:** Manizheh Karami **E-mail:** karami@shahed.ac.ir

is made of only one cell, as a eukaryotic animal, does all vital actions such as nutrition, excretion, respiration, reproduction and osmotic regulation by this single cell structure. So, this animal is physiologically a balanced creature and therefore it has own complexity (2). This freely living micro-organism is used as a very suitable animal model in neurophysiology studies (3, 4).

Today, electromagnetic devices are widely used in human daily life, and affect the growth and development process so we need to study their effect on the body (5, 6). Low frequency electromagnetic fields have been shown to alter the electrolyte nature of body fluids and cells (7). These fields affect calcium (Ca) channels, known as voltage-sensitive channels, causing changes in the intracellular Ca concentration and subsequently changes in the activity of enzymes, especially kinases (8), which are responsible for gene expression. As a result, protein synthesis, division, reproduction, and behavior are altered in a manner similar to hormones and secondary messengers. It has also been shown that the phenomenon of apoptosis can be affected by the magnetic field (9, 10). Magnetic fields have also been shown to affect cell membrane properties (11).

Protection against colchicine-induced neuromotor dysfunction has also been documented in paramecia exposed to SMF (12). *P. caudatum,* a valuable eukaryotic organism with local availability and opportunity for low-cost laboratory cultivation, has been shown to have a complex motor system sensitive to colchicine (1), an antimitotic agent with specific motor function defects (12). With the idea that the magnetic field can reduce locomotor disturbance through Ca channel conductance, we looked for the protective mechanism of the magnetic field in *Paramecium* in the presence of a Ca channel blocker, MgSO4, to rule out the mechanism of Ca involvement.

2. Materials and Methods

2.1. Animals

The animal that was studied in this research was *P. caudatum*, which was collected from the temporary ponds around Shahed University campus. Specific identification of the animal after collection was conducted by diagnosis kits. In the next step, the animals were cultivated in straw and then passaged successively and weekly in order to achieve a rich and pure environment. This cloning took almost two months.

2.2. Used materials

MgSO4 was provided (Merck, Germany) and used at doses of 1, 4, 8, 16 and 32 µg/µL, Silver nitrate (Merck, Germany) was freshly prepared and used as a Fast kit after sonication, Lugol's iodine (freshly prepared iodine-potassium iodide solution), light green dye and Feulgen's dye were purchased from Arman Co., Tehran, Iran.

2.3. Sampling

A sample (0.1 mL) of the pure culture was pipetted onto a slide with a Pasteur pipette and studied under x40 magnification of a photomicroscope (Olympus, Japan) for 30 seconds at 5 seconds intervals. In each experiment, sampling was repeated 25 times, later the mean was calculated and used in the analysis.

2.4. Preparation of natural culture medium for *P. caudatum*

First, half a liter of water was heated. After boiling, a handful of straw was added and boiled for 5 minutes. Then, after cooling, it was poured into a dark-colored jar and stored in a refrigerator (4°C) and used for newly cultured cells if needed.

2.5. Animal growth

According to the present observations, the time required to reach the Log growth phase for *Paramecium* was about 7-10 days, which was affected by factors such as light and temperature. Therefore, fresh cultures were passaged in 7-day periods and this process was continued weekly for about two months until a pure culture was obtained.

2.6. Behavioral studies of *P. caudatum* in magnetic field and non-magnetic field samples

Animal movement is an important criterion for the study of *Paramecium*. One of them is s-shaped swimming, which was counted in 5-second intervals in 30 seconds in each sampling. We sampled repeatedly (25 times), so the data was averaged with minimized error to represent the movements.

2.7. Investigating the behavior of *P. caudatum* in the control group exposed to the magnetic field (72 hours) and outside the field (normal magnetic conditions or laboratory)

First, a volume equal to 0.1 mL of *Paramecium*-

rich culture medium was transferred to a glass slide using a Pasteur pipette and placed under a light microscope at x40 magnification. The number of spiral sigmoid movements (s-shaped movement) of *Paramecium* under that view in the presence of 1 µL of distilled water was counted for 30 seconds with intervals of 5 seconds, and after 25 repetitions, it was recorded as the result of the control group.

2.8. Investigating the movement behavior of *P. caudatum* after injecting different doses of magnesium sulfate in the magnetic field and laboratory samples

In order to investigate the effect of magnesium sulfate (1, 4, 8, 16 and 32 µg/µL) on the movement behavior of *Paramecium*, in the first step, a volume equal to 0.1 mL of the desired culture medium (one sample) was prepared. It was transferred onto a glass slide and observed under a light microscope at magnification. In the next step, 1 µL of the substance was added to the sample using a Hamilton syringe. The spiral movements of the animals were recorded simultaneously for 30 seconds at 5-second intervals. This protocol was carried out for both the field group (72 hours) and the group placed in the laboratory environment.

2.9. Light green staining

The light green dye (0.1% aqueous solution in distilled water) was stirred, filtered through filter paper, and poured into a small beaker. It was used to examine the nucleus of the cells. For staining, sampling was first performed, and after the sample was fixed (dried in air), 1% light green dye was added to the sample.

2.10. Nucleus staining with Feulgen reaction method

This reaction is a quantitative check for the nucleus. The sample was exposed to hydrochloric acid (60°C) for 5 seconds and then exposed to prepared Feulgen stain in an incubator set at 37ºC for 90 minutes. The sample was washed once with sodium metabisulfite, then briefly (2-3 seconds) exposed to light green (0.01% aqueous) and finally dehydrated with ethanol, cleared with xylene and mounted with Entellan (Merck, Germany). The DNA was stained red. The background was light green (almost colorless).

2.11. Specific staining for neuromotor and calcium channels

To identify the neuromotor system and Ca channel using the Fast kit, we first removed a volume of 0.1 mL of the culture medium with a pipette and placed it on a glass slide and studied it under fixed magnification (x40). After fixation (drying in the air) coloring was done, which included four steps: Fast silver kit (xylol + 10% formaldehyde + 0.1% AgNO3) was poured on the slide after ultrasound (to ensure homogeneity and no precipitation) and incubated at 37°C for 30 minutes. After this step, the enhancer (formaldehyde 10% + NaCO3) was added to the slide and placed under a wire lamp (100 watts) for 35 minutes. Then, stop solution (CaNa2, pH=5.5) was poured onto the slide and left for 10 minutes. The slide was then washed with distilled water for 5 minutes, then dehydrated with alcohol for three minutes, and then cleared twice in xylene for three minutes each. Finally, Entellan was added to the slide and covered with a coverslip.

2.12. Staining method to detect the motor nervous system (silver line) using dry silver nitrate

First, using a pipette, a volume of 0.1 mL of the culture medium was removed using a pipette and placed on a glass slide. After drying (fixation), a 1% silver nitrate solution (0.1 g AgNO3 dissolved in 10 mL of distilled water) was poured on the slide. The slide was then placed in complete darkness for 7-10 minutes and then washed in tap water and distilled water. Then, we placed the slide under direct sunlight for 20 minutes. Then we placed the slide in alcohol and xylene for three minutes each and glued it.

2.13. Staining method for detection of caudal tuft of cilia of *P. caudatum*

To stain the long cilia at the end of the tail of *Paramecium* (known as caudal tuft of cilia), we first removed a volume of 0.1 mL of the culture medium with the help of a pipette and placed it on a slide. Then, we added a volume of one microliter of Lugol's stain with a Hamilton syringe and then examined the long cilia (caudal tuft) properly under a microscope. To examine the tuft at 40 and 100 magnifications, we placed a cover slip on the slide to prevent damage to the microscope lens.

2.14. Static magnetic field (SMF)

The magnetic field (0.061 T) was prepared by Dr. Abazar Hajnorouzi in the nanotechnology laboratory of Shahed University in the form of a ring permanent magnet made of ferrite with a thickness of 2 cm and an inner diameter of 6 cm.

2.15. Statistical calculations

Raw outputs were analyzed using SPSS software (version 22). First, the normal distribution of the data was checked with the Kolmogorov–Smirnov test, and if confirmed, a parametric test (analysis of variance; ANOVA) was performed. If significant, the Tukey's test was followed and the difference between groups was examined. $P<0.05$ was considered significant.

3. Results

3.1. Specific identification of *P. caudatum*

P. caudatum was identified by several key features, including the presence of a longer caudal tuft of cilia at the end of the body, a bean-shaped macronucleus, and a small compact micronucleus surrounding the macronucleus. The position of a curved oral groove and the silver line (neuromotor) system are also unique and have been studied (Figures 1-3).

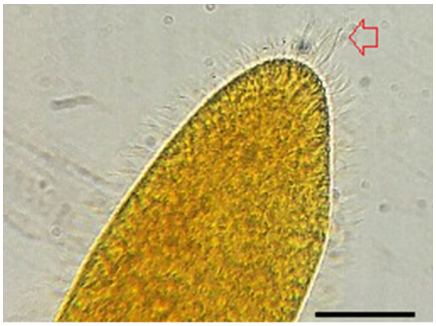


Fig. 1. Image of *P. caudatum* for species identification. Lugel's staining was performed to show the long cilia at the end of the tail (red arrow). The caudal tuft is a specific characteristics of *P. caudatum*. Scale bar is 50 µm.

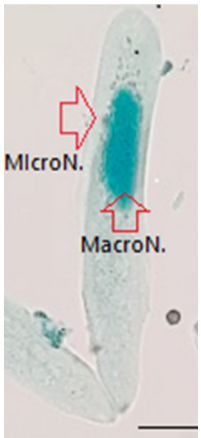


Fig. 2. Image of a *P. caudatum* nucleus stained with light green for species identification. Micro and macronuclei of *P. caudatum*, which are characteristic of this animal were shown: a bean-shaped macronucleus and a compact micronucleus surrounding the macronucleus. The scale is 50 µm.

Fig. 3. Image of a *P. caudatum* stained with the Fast kit silver nitrate method for species identification. The curvature of the Oral Aperture structure is visible. This. The scale is 50 µm.

3.2. The growth curve of *P. caudatum*

Figure 4 shows the growth curve of *P. caudatum*. The growth of the animals within 15 days after cultivation is consistent with the growth curve pattern of *P. caudatum* (Figure 4).

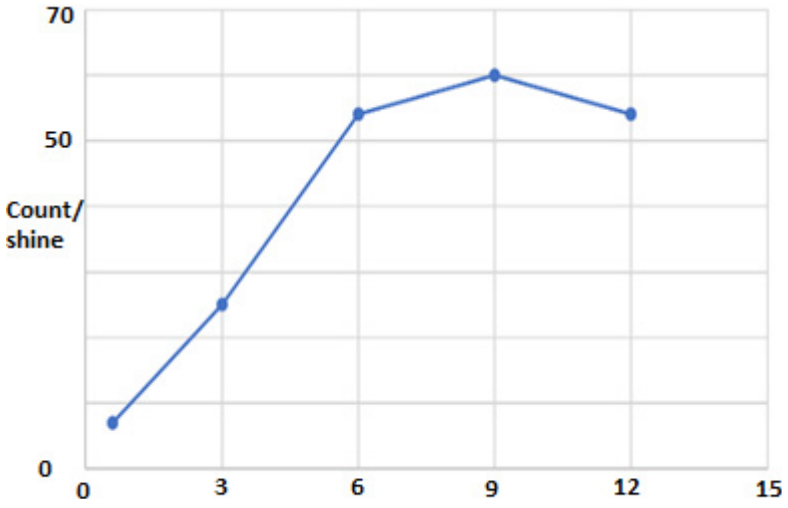


Fig. 4. This figure shows the growth curve of *P. caudatum*. The growth of the animals is shown over a period of 15 days after cultivation. The view (shine) under a four objective lens of a light microscope shows approximately 1/66.66 mL of the sample volume. Therefore, by multiplying the mean count by 66.66, the total number of cells in one mL of culture medium can be obtained. As is clear from the graph, after an optimal period, the number of animals decreases.

3.3. The intensity of the applied magnetic field

First, the north and south poles of the magnet used were measured by a special device, and then its magnetic field intensity was determined by a Teslameter (0.061 T). At the center of the ring

magnet, this intensity was constant and was used to prepare an experimental group for this study (field group), which remained in the field for 72 hours (Figure 5).

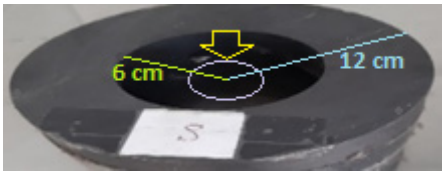
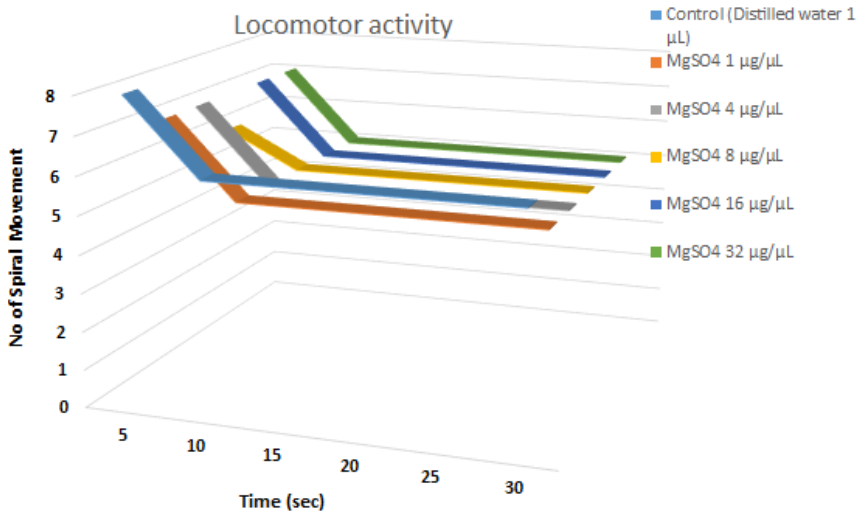


Fig. 5. The image of the magnet used in this study. It was provided by Dr. A Hajnorouzi. The arrow indicates the location of the field sample placement. The inner and outer diameters are also shown. They are two ring magnets (each 2 cm thick).

3.4. The effect of different doses of magnesium sulfate on the locomotor activity of animals in the field and outside the field (Laboratory with normal geomagnetic condition)

The number of spiral movements in a 30-second time period at 5-second intervals is shown under objective lens 4 with a comparative view between the two groups (SMF or, for short, field and Lab). The result is a reduction in movement, but under normal conditions, it reached a new equilibrium after some time, but in the field group, it was more severe and had large fluctuations and was somewhat dose-dependent (Figure 6).



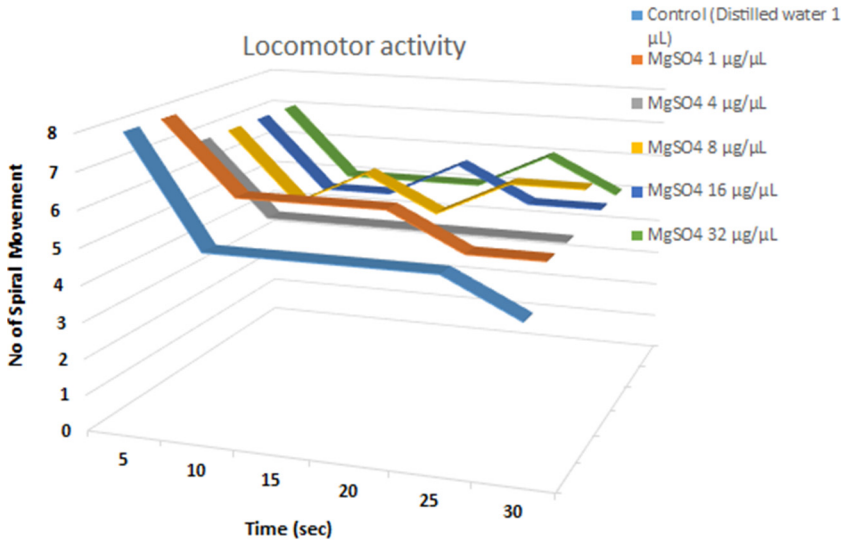


Fig. 6. The number of s-shaped spiral movements in a 30-second time period at 5-second intervals for the groups (Lab and SMF) in the MgSO4 treatment. The effect of different doses of magnesium sulfate (1, 4, 8, 16 and 32 µg/µL) on the motor activity of animals was compared with the corresponding control and had a decreasing effect, but it was more significant in the SMF+MgSO4 group.

3.5. Effect of different doses of magnesium sulfate on avoidance response in magnetic field and Lab samples

The avoidance response in *P. caudatum* in the presence of different doses of magnesium sulfate was statistically analyzed and shown as a percentage in Figure 7. According to these results, this response was dose-dependent and occurred significantly at higher concentrations. It should be noted that the speed of movements was higher at higher doses. At low doses (1 and 4 µg/µL), the percentage of avoidance response was very low (about 7%) and was not significant compared to the control group. At higher doses (8, 16, and 32 µg/µL), the percentage of avoidance responses was significantly higher than in the control group (28% at 8 µg/µL, 43% at 16 µg/µL, and 67% at 32 µg/µL).

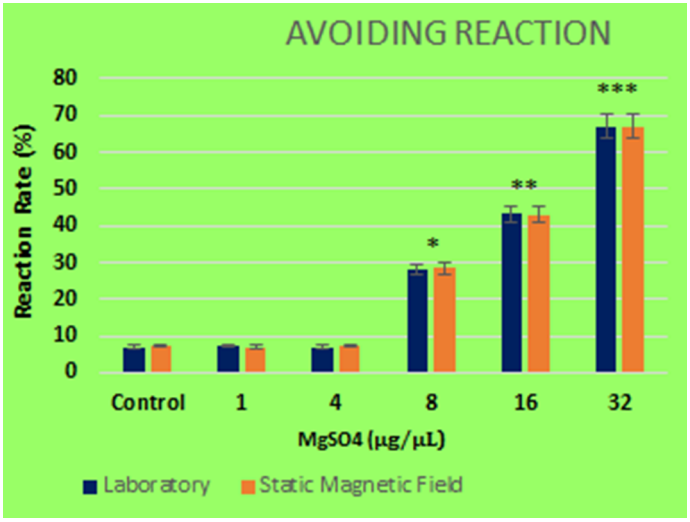


Fig. 7. Effect of different doses of magnesium sulfate on avoidance. The response was dose-dependent and occurred significantly at higher concentrations of SMF/Lab+MgSO4. Stars are based on Tukey's test (*P<0.05, **P<0.01, ***P<0.001 *vs*. control).

3.6. Results of investigating the neuromotor system and Ca channels using the Fast kit method

After staining with the Fast kit method, in the presence of magnesium sulfate, observations indicate a change in the arrangement of the pellicular system and a destructive effect on this system, but the density of Ca channels did not change significantly (Figure 8).

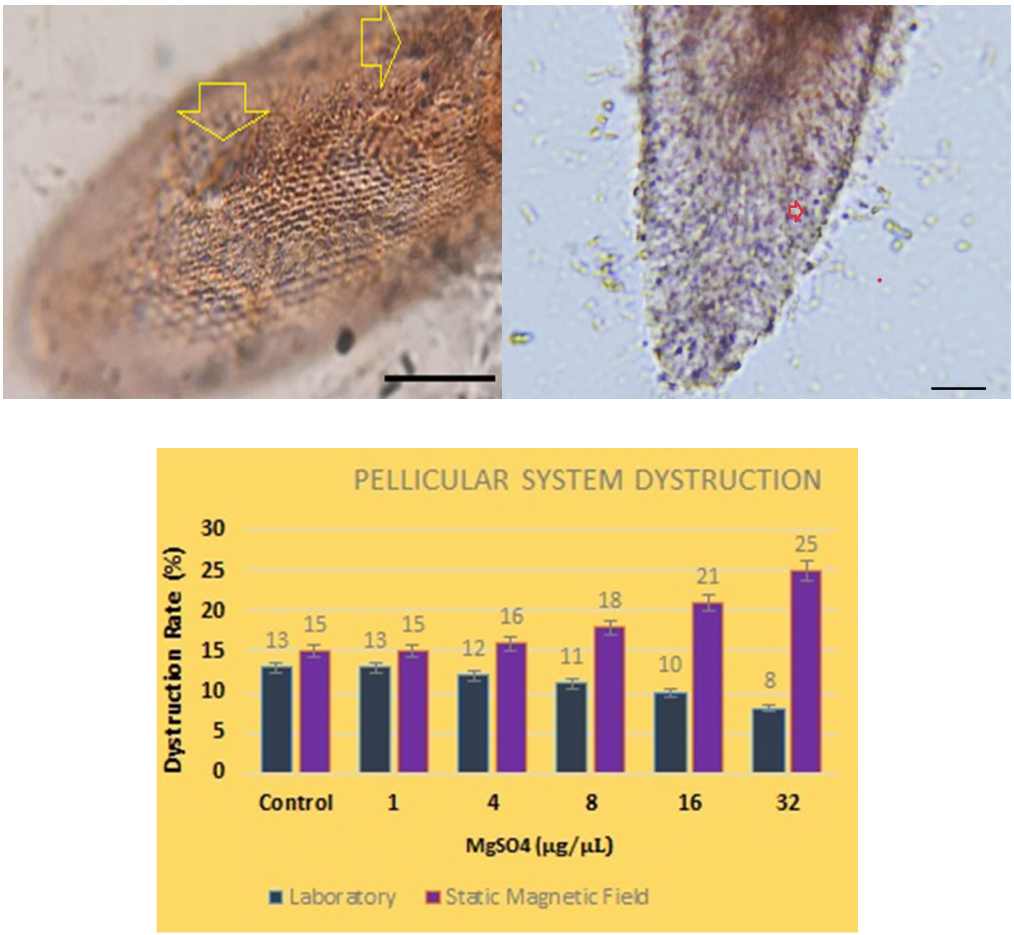


Fig. 8. An overview of the pellicular neuromotor system and Ca channels. The graph shows the percentage of degradation at high doses of MgSO4 (8-32 µg/µL) under SMF. The upper left image indicates the destruction of the pellicle system at a dose of 16 µg/µL magnesium sulfate in the SMF group, and the right image shows the Ca channel locality under a dose of 8 µg/µL *in vitro* (channels are seen at the basal surface of the cilia in the pellicular system). Sacle bar is 50 µm.

3.7. Results of *P. caudatum* nucleus staining using light green and Feulgen nuclear reaction

The nuclei of *P. caudatum* in both control and experimental samples were stained using the light green (Fast kit) and Feulgen reaction methods. Observations showed that both intact and fragmented nuclei were presented in these samples, so to obtain the nuclear destruction percentage in these samples, we calculated the proportion of fragmented cases to the total cells, which represented 29% of field samples and 24% of Laboratory samples (Figure 9).

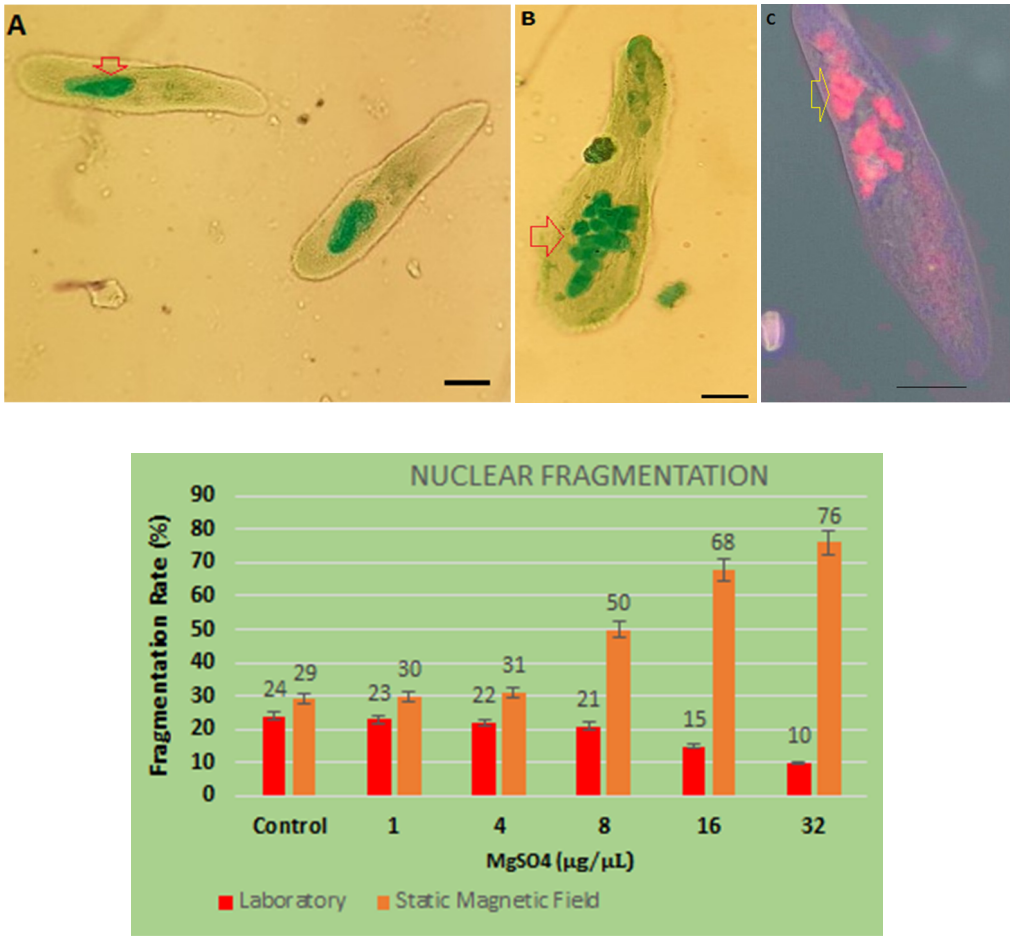


Fig. 9. Nuclear fragmentation in experimental (B: Light green, and C: Feulgen nuclear reaction) compared to the control (A). Fermentation in SMF samples is higher than in the laboratory. Scale = 50 µm.

4. Discussion

Our aim in this research was to investigate the effects of Ca channel blocking by MgSO4, assuming that the magnetic field can protect *P. caudatum*. The effect of different doses of MgSO4 magnesium sulfate on locomotor activity behavior and avoidance response after exposing animals to magnetic field was recorded and compared with laboratory samples. The decrease in the number of spiral movements of paramecia may reflect the destruction of the microtubule system in the cell membrane and the decrease in cilia activity, which ultimately causes a decrease in movements in paramecia. *Paramecium* is sensitive to environmental stimuli such as heat, cold, mechanical stimuli, etc. and reacts to them. When an animal is exposed to electric and magnetic fields, it reacts in a way known as avoidance. It has been previously documented that exposure to a magnetic field can protect this animal from noxious stimuli (13). Trichocyst discharge is another defense response that *Paramecium* displays against a noxious stimulus (14). Our aim was to show whether there is a relative protective effect on motility by SMF in paramecia.

Previous evidence has shown that Ca ions play an important role in the protective effect of the magnetic field, as it leads to greater conductance of membrane Ca channels and increased excitability and response to external stimuli in *Paramecium* (15). Previous findings have also shown that low magnetic field intensity reduces Ca uptake in rat lymphocytes by affecting cytochrome P-450 (16). In this research, the Ca channel was inhibited by MgSO4, but the electrolyte changes on both sides of the *Paramecium* membrane were not

investigated, but the changes in the concentration of the Ca channel blocker MgSO4 had a significant effect, which is consistent with the findings of others. *Paramecium* had an avoidance behavior when dealing with MgSO4, so that it performed this behavior at a higher speed in high doses (dose 8 µg/µL and more). During the previous research conducted by Daryl, after adding KCl to the culture medium of *Paramecium*, the result was an avoidance reaction at high doses of this substance and the behavior was related to a change in the pellicular system or a change in the nucleus or a change in membrane potential (17). Therefore, the response of *P. caudatum* to stimuli is due to changes in the structure of the neuromotor system of the animal's pellicle. This system is an integrated system that can be seen in some organs such as nerve cells and ciliated leukocytes, and it can be seen using a microscope and the Fast kit staining method.

After staining with Fast kit and checking the degree of destruction, we showed that magnesium sulfate alone had a less destructive effect on the cytoskeleton of *P. caudatum*. But when *Paramecium* was exposed to magnetic field, the rate of degradation increased, so it seems that the combination of two agents (Ca blocker and SMF) cannot have a protective effect on the cytoskeleton and shows contradictions with some statements about the advantages of a SMF (18), because the rate of degradation of the pellicular neuromotor system at 32 µg/µL in the field was 25% but at the same dose outside the magnetic field (Lab) it was 8%. In agreement, one study has demonstrated that Fe2O3 nanoparticles alone had a protective effect on colchicine-induced neurotoxin degradation on *Paramecium* neuromotor and animal locomotion, but this effect was reduced by applying a magnetic field (12). Some other researchers have provided information about the harms of higher intensity magnetic field by investigating the biophysical mechanisms (19). In a study that investigated the protective effect of Ca channel blockers on motor learning deficits in mice exposed to very low frequency electromagnetic waves (1 mT, 50 Hz), the results showed that mice treated with the Ca channel blocker, amlodipine, had an enhanced motor learning and concluded that the deficit is partly due to increased Ca levels in certain parts of the mouse brain such as the hippocampus and brainstem (20, 21).

In another research, it has been stated that electromagnetic fields can interact with biological tissues and have positive and negative effects on cell viability. In excitable cells, exposure to electromagnetic fields induces Ca influx through voltage-gated Ca channels, leading to cell activation and an antioxidant response. Upon further activation, oxidative stress causes DNA damage or cell death (11, 22). In another previous experiment, a static magnetic field (SMF) with an intensity of 10 T in the presence of X-rays caused an increase in the formation of micro nuclei and damage to cellular DNA. Also, the presence of iron ions in the cell culture medium along with the magnetic field increased DNA damage (23). Therefore, the synergy of SMF and ionizing radiation should be further investigated (24).

In the present study, after staining the nuclei with bright green color or Feulgen reaction, the nuclei of the group treated with different doses of MgSO4 were studied and compared with the control sample. Nuclear degradation was dependent on blocker doses, so that at low doses (samples that were in the magnetic field for three days), the percentage of nuclear destruction was not significant compared to the control group, but with increasing MgSO4 dose (from 8 µg per µL and higher), the cells had more nuclear destruction and the percentage of destruction was significantly increased compared to the control group, so that at the dose of 32 µg/µL in the SMF samples it was approximately 76%.

The ciliate has two types of nuclei in its normal cytoplasm: micronucleus and macronucleus. These two types of nuclei are different in terms of structure and function (25). The micronucleus is a normal diploid nucleus and is involved in sexual reproduction: The animal undergoes meiosis, which leads to the formation of the gamete nucleus, and after the fertilization of the haploid gamete nuclei, the zygote nucleus is formed, and this synkaryon creates both micro- and macro-nucleus (1, 26).

It is the damage to the macronucleus that impairs the movement of the animal, so in this study, damage to the nucleus corresponds to damage to the structure of the motor neuron, and no recovery effect was observed for this part under field exposure.

Conclusion

In this research, a simple animal model (*P. caudatum*) was used, which was first identified (1, 27-30) and grown (31) according to the sources. Then, the effect of static magnetic field (SMF) on *P. caudatum* was investigated under Ca

channel blockade with MgSO4. Under Ca channel blockade, the effect of SMF on *Paramecium* motility and structure worsened, a finding suggesting that Ca ion conductance plays a role in the protective effects of the SMF.

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Conflict of interest

The authors of this article have no conflict of interest.

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