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Investigating the effect of magnetic field and magnetite nanoparticles on the toxicity caused by colchicine in *Paramecium caudatum*

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Abstract

Introduction and purpose: *Paramecium caudatum* is a suitable animal model. We studied effect of magnetite nanoparticles under static magnetic field (SMF) on the toxicity of colchicine in *P. caudatum*. Materials & Methods: The animal was collected from nature's water resources, and cultivated in the laboratory weekly with straw to purify. The pure culture was divided into two groups, one in the laboratory and the other group under SMF (72 hours). Each group was divided into control (1 µL of distilled water), colchicine alone (doses of 1, 3 and 9 µg/µL), nano Fe3O4 alone (doses of 1, 3 and 9 µg/µL) and nanomaterials + colchicine. A sample at a volume of 0.1 mL was placed on the slide and studied 25 times under x4 in a 30-second period (with 5-second intervals). Animal's s-swimming movement was counted for 25 times, within the 30-second. Also, the neuromotor system, the macronucleus, and the avoidance reaction were studied and data were computed using ANOVA under 0.05 error. Results: The field singly had a little reduced effect on the motility of *Paramecium*. Colchicine decreased locomotion in both groups. In addition, it showed destruction of macronucleus and pellicle and most importantly destructed the neuromotor system, but no significant difference was observed in tubulin density. In the case of Fe3O4 NPs + colchicine, a little protective effect caused by nanomaterials was observed. Conclusion: The effect of colchicine as a neurotoxin is focused on the motor system, and Fe3O4 NPs has little protective effect regardless of laboratory and SMF.

1. Introduction

Paramecium is found in ponds and semi-stagnant waters containing abundant aquatic plants and decomposing organic matter. It has 150 to 300 µm long but has lost its symmetry due to the mouth groove located on one side, which extends diagonally toward the back. Its pellicle is membranous, colorless and flexible, which is covered by cilia arranged in longitudinal rows (kineties). This animal has two nuclei that are visible only if the cell is stained. *Paramecium* is holozoic and feeds on bacteria, algae and other small organisms. It is also able to change its swimming speed and strategy [1].

Colchicine a botanical alkaloid derived from the flower *Colchicum autumnale* L. was originally used to treat gout for centuries and is used in the treatment of familial Mediterranean fever and cancer. The crucial mechanism of action of colchicine is tubulins disruption, which are key components of the cytoskeleton (αβ-tubulin heterodimers). Mechanistically, it inhibits tubulin polymerization, and impairs several processes including mitosis, intracellular transport, and phagocytosis. This leads to subsequent down regulation of multiple inflammatory pathways and modulation of innate immunity, so it t has also been used in other diseases including Behcet's disease (BD), pericarditis, coronary artery disease and other inflammatory and fibrotic conditions [2]. However, it has been reported that combining colchicine with statin treatment might increase the risk of myalgia and, very rarely, acute rhabdomyolysis, especially among patients with renal impairment [3]. This small molecule with a molecular weight of 399 Da due to long half-life of elimination can inhibit cell division by fixing the intracellular tubulin and disrupting the mitosis and transport systems. Thus, the organs with active cell proliferation, such as the gastrointestinal tract, bone marrow, hair follicles, and granule cells, the major principal cell type in the dentate gyrus [4] are susceptible to colchicine toxicity. However, the cell damage caused by colchicine cannot be resolved with conventional treatment, but based on the biomechanical properties of the cells, appropriate methods should be adopted [4-5].

The use of metal NPs (MNPs) and static magnetic field (SMF) in removing the harmful effects of this neurotoxin has previously been conducted [6]. In this research, we aimed to test magnetite (Fe3O4) NPs in the presence of SMF. MNPs, can be provide by electrochemical method [7-8]. It is important to know whether one or both of these interventions protect against colchicine toxicity. Because colchicine poisoning has been shown to be an uncommon but serious form of drug poisoning and may cause systemic complications and death. In toxic doses, it causes nausea and vomiting and bone marrow suppression, which often leads to sepsis, hypocalcemia, adult respiratory distress syndrome, and direct effects on the heart [9].

In this study, the effect of magnetite and/or SMF on colchicine-induced microtubule toxicity in *Paramecium* was investigated.

2. Material & Methods

2-1. Animal subject

The animal studied in this research is a single-celled protozoan *Paramecium caudatum*. This animal was collected from natural water sources and its species was identified using key features. Then it was cultured with straw and to prepare a pure rich culture of this animal, it was cultured repeatedly in a natural way (passage was done weekly). This method is very valuable and affordable compared to the artificial culture medium, and thanks to it, we reached a rich and pure environment of this animal. All ethical principles of work have been followed according to the Declaration of Helsinki (DoH) and the code of ethics has been granted to this research by the ethics committee of the university (IR.SHAHED.REC.1402.011).

2-2. Materials used

Magnetite nanomaterial (Fe_3O_4 NPs) (prepared by Dr. A Hajnorouzi, Nanotechnology Lab., Shahed University), Colchicine (Sigma, U.S.), Silver nitrate (Merck, Germany), NADPH (Merck, Germany), NBT (Merck, Germany), Hematoxylin (purchased from F Arman Co., Iran), Tubulin beta specific antibody (Germany, Santa Cruz Biotechnology, Inc.), Entellan glue (Merck, Germany).

2-3. Method of preparation of natural culture medium of *Paramecium*

To prepare the natural culture medium of *Paramecium*, half a liter of tap water was heated to the boiling point. Then we added about 10 g of dry straw to boil with water. After about 5 minutes, the boiling liquid will turn yellow. Then we removed the glass from the heater and divided its contents into glass containers. We waited for a while until the temperature of the liquid reaches the ambient temperature and cools down. Then, with the help of a Pasteur pipette, we took some of the previous culture samples containing *Paramecium* and poured them into new containers and covered glass containers with porous aluminum foil. The single-celled animal divides and reproduces very quickly. The maximum population was obtained about five to seven days after transfer to fresh culture medium.

2-4. Behavioral studies (motility measurements) of *Paramecium*

Paramecium has several stereotyped locomotor behaviors. One of them is spiral or s-shaped movements, so that each spiral movement can be considered as a number of swimming movements.

The s-shaped movements of this unicellular animal can be observed at 5-second intervals for 30 seconds under a fixed field of view of a light microscope provided at x4 magnification. The number of these movements in each interval was recorded and then statistically calculated, but this part of the study (counting sigmoid movements during the 30 second sampling period) was repeated 25 times to minimize the error. It should be noted that for each dose or control group, the volume of each sample was 0.1 mL and the microscopic surface was about 1600 µm. These raw data were analyzed in SPSS software (version 22).

2-5. Investigating the movement behavior (spiral movement count) of *P. caudatum* in control and experimental samples

A sample volume of 0.1 mL was transferred from each pure stock medium (one in a laboratory environment with normal geomagnetic conditions and the other in a static magnetic field (SMF) of 0.061 Tesla for 72 h) using a pipette. The slide was observed under a light microscope with x4 magnification at 30-second time periods with 5-second intervals, repeatedly (25 times). The slide was then kept in the laboratory to be fixed and used for future cellular-molecular studies. Since the substances studied in this research had to be inoculated into the sample with a volume concentration of 1 µL, therefore, with the help of a Hamilton syringe, one µL of distilled water (in the case of control) or colchicine (1 to 9 µg/µL) /Fe_3O_4 (1 to 9 µg/µL) was inoculated into the sample.

2-6. Staining with silver nitrate (fast kit)

0.1 g of silver nitrate was dissolved in 10 mL of distilled water to obtain a 1% silver nitrate solution. Then it was homogenized with the help of an ultrasounicator machine. Fixed samples were studied immediately or at appropriate times with this method to visualize the neuromotor system. After sonication, the stain was immediately added to the dried samples and the slides were placed in a dark environment away from any light for 10 minutes. After that, the samples were exposed to direct sunlight for 40 minutes, but at the same time, the slide was kept wet. A brown deposit appeared on the slides, which we washed with a dropper to remove the deposit. Then we put the slide in a container full of distilled water for 10-20 seconds. The slides were then transferred to 50, 70 and 96% alcohol (each for 15-20 seconds) to dehydrate. Then they were placed in xylene for 2-3 minutes (2 times). Immediately after removing the slides from the xylene, Entellan glue was poured on the samples and then coverslipped. About two days later, the slide was studied with a light microscope. This method shows the silver line system or neuromotor system of the animal, which is a very complex and microtubular structure.

This method is very similar to the Golgi staining method for visualizing nerve branches, and so this experiment teaches everyone why this animal is a valuable model of a moving neuron!

2-7. Methylene blue staining of pellicle

Methylene blue 0.1 g was dissolved in 100 cc of distilled water to obtain a colored solution of 0.01%. To remove sediments from the solution, it should be filtered three times with filter paper and stored in a dark glass. The fixed slide was passed through xylene and 96% alcohol, 70% alcohol, and 50% alcohol for 15-30 seconds. After that, the slides were passed through a container filled with distilled water for 10-20 seconds. In the next step, the slides were placed in the methyl dye solution for 30 to 45 minutes and then washed in the sink using the dropper method. Then they were placed in a glass full of distilled water for 10-20 seconds and then the slides were transferred to 50, 70 and 96% alcohol (each for 15-30 seconds) to dehydrate. Then they were placed in xylene for 3-5 minutes. After removing the slides from xylene, Entellan glue was poured on the samples and then slipped. About two days later, the prepared samples were studied with a light microscope.

2-8. Nucleus staining with hematoxylin

The 20% hematoxylin stock should first be diluted to the appropriate concentration by adding a little distilled water. Fixed slides were immersed in xylene, 96% alcohol, 70% alcohol, and 50% alcohol for 3-5 minutes. After that, the slides were passed through a container filled with distilled water for 10-20 seconds. In the next step, prepared hematoxylin dye was added to the slides. After 30-45 minutes, the slides were washed in the sink using the dropper method and then placed in a glass filled with distilled water for 10-20 seconds. Slides were then transferred to 50, 70, and 96% alcohol (15-30 seconds each) to dehydrate and then embedded in xylene for 2-3 minutes (2 times). Then mounted with Entellan. About two days later, the prepared samples were examined with a light microscope.

2-9. Examining calcium channels with antibodies

Fixed samples were immersed in distilled water for 10-20 seconds. In the next step, antibody was added to the slides. After 10 minutes, HRP (horseradish peroxidase) and then bovine antiserum were added to the slides. Then the slides were washed in the sink using a dropper method and placed in a container full of distilled water for 10-20 seconds. They were placed in counterstain (haematoxin) for 5-10 seconds. After that, the slides were transferred to distilled water, 50, 70 and 96% alcohol (each for 15-30 seconds) to dehydrate and then placed in xylene for 2-3 minutes (2 times). Assembly was done with Entellan. After about two days, the prepared samples were studied with an equipped microscope.

2-10. NADPH-diaphorase for marking nitric oxide synthase activation

Nitrogen monoxide, nitric oxide (NO), is a colorless gas. This molecule is a free radical and is considered as an important intermediate messenger in physiology. In mammals such as humans, NO is an important molecule in cell signaling that is involved in many physiological and pathological processes. This compound has vasodilation properties and has a short half-life in the blood (a few seconds). Low levels of NO production are important in protecting organs such as the liver from ischemic damage. It is a key biological messenger of vertebrates that plays an important role in biological processes. This known biological product is present in almost all types of organisms, including bacteria, plants, fungi, and animal cells such as *Paramecium*.

This substance, known as an endothelium-derived relaxing factor (EDRF), is synthesized from L-arginine, oxygen, and NADPH by NO synthase (NOS) enzymes. NO is highly reactive (lifetime of a few seconds), but diffuses freely across the membrane. By inhibiting vascular smooth muscle contraction, platelet aggregation and leukocyte adhesion to the endothelium, it contributes to vascular homeostasis. Individuals with atherosclerosis, diabetes, or hypertension often exhibit defects in NO pathways [9].

To start staining, about 0.4 mg of NBT and 2 mg of β-NADPH were dissolved in 10 mL of distilled water and stored separately in the refrigerator. Fixed slides were transferred to xylene, 96% alcohol, 70% alcohol, and 50% alcohol for 2-3 minutes. They were suspended in distilled water containing 1-2 drops of Triton X 100, and in the next step, NADPH and NBT solution were added to the slides in a ratio of one to one. After a period of 45-60 minutes, the slides were placed in a container filled with distilled water for 10-20 seconds and then transferred to 50%, 70% and 96% alcohol respectively (each for 2-3 minutes). Then they were placed in xylene for 2-3 minutes (2 times). After removing the slides from xylene, Entellan glue was poured on the samples and then coverslipped.

2-11. Examination of the caudal tuft of the cilia of *P. caudatum* with Lugol

Lugol's solution was used to examine the terminal cilia *of P. caudatum*, which are called caudal tufts. This process was done to determine the species.

We took a drop of the *Paramecium* culture medium with the help of a pipette and transferred it to the surface of the slide. We paused for 5 seconds to acclimate each cell to the new environment. Then, one microliter of Lugol was added to the sample with a Hamilton syringe, and after a few seconds, we covered the slide and studied it under the microscope.

2-12. Statistical calculations

All data were analyzed using Kolmogorov-Smirnov test and analysis of variance (ANOVA). Subsequent analysis was performed with Tukey. α was 0.05. Also, the qualitative analysis (images) was converted to quantitative with the help of Image J software (free Java).

3. Results

3-1. Determination of *P. caudatum* species

This species has special characteristics that distinguish it from other *Paramecium* species. It has a caudal mass of cilia at the end of the body (caudal tuft) and has a bean-shaped macronucleus and a compact micronucleus located in the umbilical depression of the macronucleus (Figure 1). Also it has a special oral apparatus.

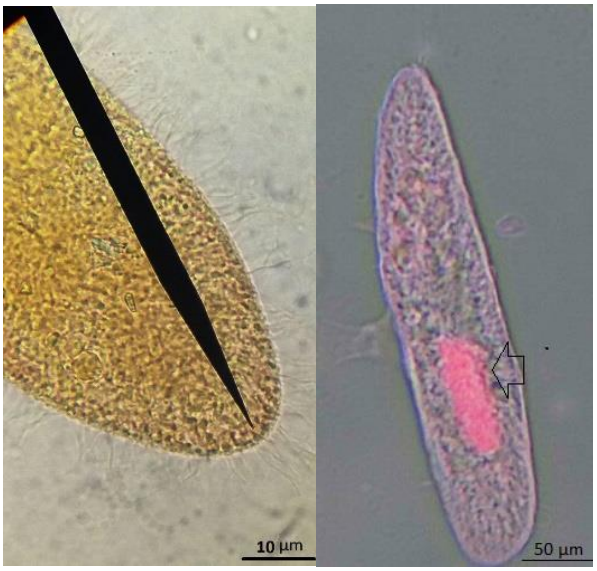

Fig. 1. *P. caudatum* photographed for species identification. The tail cilia at the end of the cell body is one of the characteristics of *P. caudatum* species. The painting is done with Lugol. The nuclei of *P. caudatum* is another characteristic of *P. caudatum* species. The nuclei were stained with hematoxylin. The macronucleus is bean-shaped and the micronucleus is located in the umbilical depression of the macronucleus. Scale bar is shown.

3-2. The single-cell growth curve

The growth curve of P. caudatum is plotted, the number of individual cells is shown by random counting of 10 views (shines) with a light microscope lens (x4). The experiment was repeated many times and then the average count was presented. The first day of cultivation until the next 12 days are shown, which corresponded to the growth curve of *P. caudatum* (Figure 2).

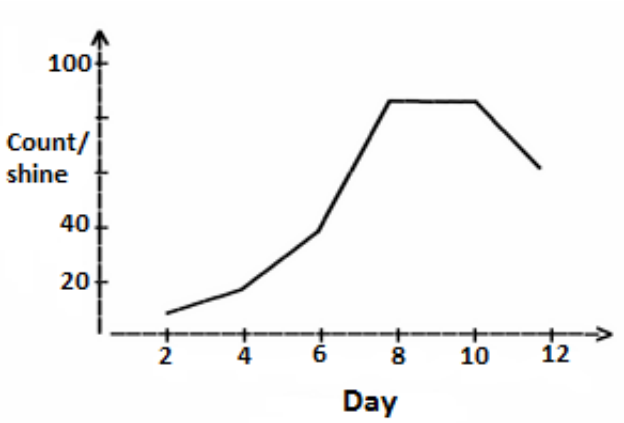

Fig. 2. The mean count/shine over about two weeks is shown. A view under the x4 lens of a light microscope shows approximately 1/66.66 mL of sample volume. Therefore, by multiplying the average count by 66.66, the total number of cells in one mL of culture medium can be obtained. After an optimal period (preferably 7 days), the number of animals decreases.

3-3. Silver-line system in *P. caudatum*

The cytoskeleton of *P. caudatum* was studied by silver nitrate staining method in order to identify the safety of the motor neuron structure or the damage caused by exposure to the substances (Figure 3). This damage occurred due to exposure to colchicine, but occurred less in the presence of nanomaterials and magnetic fields. (Figure 3).

Fig. 3. Cytoskeleton of *P. caudatum* by silver nitrate staining method (A). Damage from exposure to colchicine can be seen (B). However, it was less in samples that were exposed to nanomaterials and field (C) (Figure 3).

3-4. Locomotor activity of *P. caudatum* after receiving different doses of colchicine in laboratory and field samples

As shown in Figure 4, the effect of different doses of colchicine on the animal's locomotor activity in 30 seconds was investigated and a dose-dependent decrease was observed.

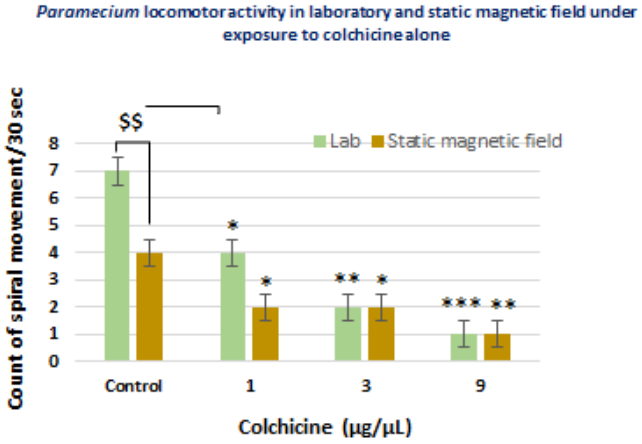

Fig. 4. Movements of *P. caudatum* after receiving different doses of colchicine in laboratory and SMF samples. All data were recorded in 30 seconds (we repeated each test 25 times and these data were averaged). A field-induced decrease in movement is observed in the comparison between the laboratory control and the field control. Asterisks are obtained by Tukey's test and indicate the difference between the experimental groups and the respective control groups (*P<0.05, **P<0.01, ***P<0.001).

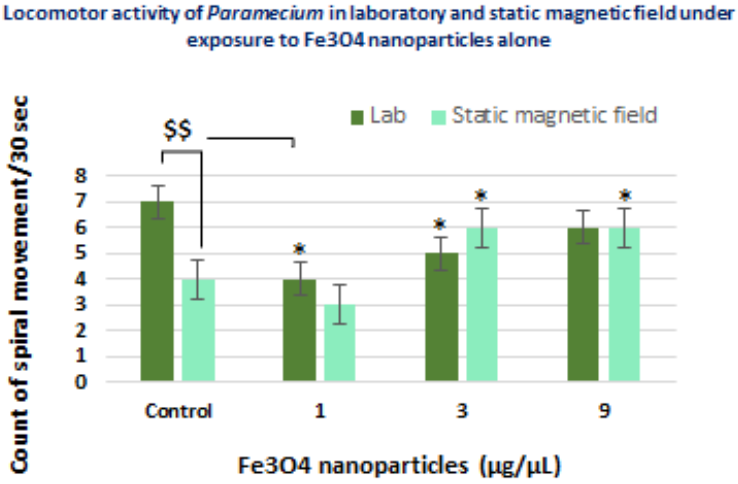

Fig. 5. The effects of different doses of magnetite nanomaterials (Fe3O4) on the locomotor activity of *P. caudatum*. The s-shaped movement of the animal was checked in 30 seconds. A field-induced decrease in movement is observed in the comparison between the laboratory control and the field control. Each test was repeated 25 times and the data were averaged. Asterisks are obtained by Tukey's test and indicate the difference between the experimental groups and the respective control groups (P<0.05).

3-5. Movement activity of *P. caudatum* after receiving different doses of magnetite nanomaterials (Fe_3O_4) in laboratory and field samples

The effect of different doses of magnetite nanomaterials (Fe_3O_4) on motor activity in 30 seconds was investigated. As shown in Figure 5, the nanomaterial under the field has relatively improved it. In the comparison between the laboratory control and the field control, a relative reduction of movement caused by the field was observed.

3-6. Investigating the effect of colchicine on the movement activity of *P. caudatum* in combined treatment with magnetite nanomaterial (Fe_3O_4) and colchicine in laboratory and field samples

Spiral movements of *Paramecium* treated with the combination of magnetite nanomaterial (Fe3O4) and colchicine was investigated. As shown in Figure 6 in the combined treatment of nanomaterial with the neurotoxin, an improvement effect was observed in the lowest dose of colchicine in combine of the highest dose of nano under the field.

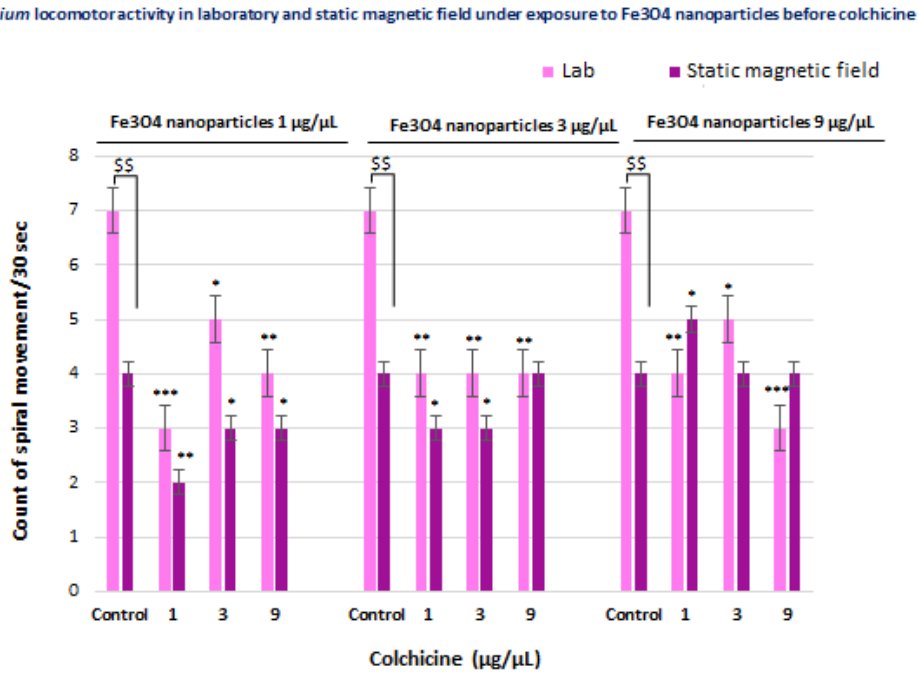

Fig. 6. Comparison of movements of *P. caudatum* treated with the combination of magnetite nanomaterials (Fe3O4) and colchicine in laboratory and field samples. Individual cells from laboratory and field samples were taken out in a volume of 0.1 mL and placed on a glass slide. Then the magnetite nanomaterials with a volume concentration of 1 µL was added to the sample. Five seconds later the colchicine was added to the sample with the same volume. The number of spiral movements of *Paramecium* was counted in 30 seconds and the average was calculated after 25 repetitions. Asterisks are obtained by Tukey's test and indicate differences between the experimental groups and the respective control groups (*P<0.05, **P<0.01, ***P<0.001).

3-7. Examination of the pellicle structure of *P. caudatum* by methyl fast staining

Animals from the control groups, colchicine alone, magnetite nanomaterials alone and colchicine + magnetite nanomaterials, in the laboratory environment and under the influence of a SMF, were studied with methyl fast method. The results show that colchicine as a neurotoxin had destructive effects on *P. caudatum* pellicle. With increasing dose of colchicine, pellicle destruction increased. And this destructive effect was less in samples treated with magnetic field. It was absent in magnetite group. In accumulative nano magnetite+ colchicine treatment a little protection was occurred (Figure 7).

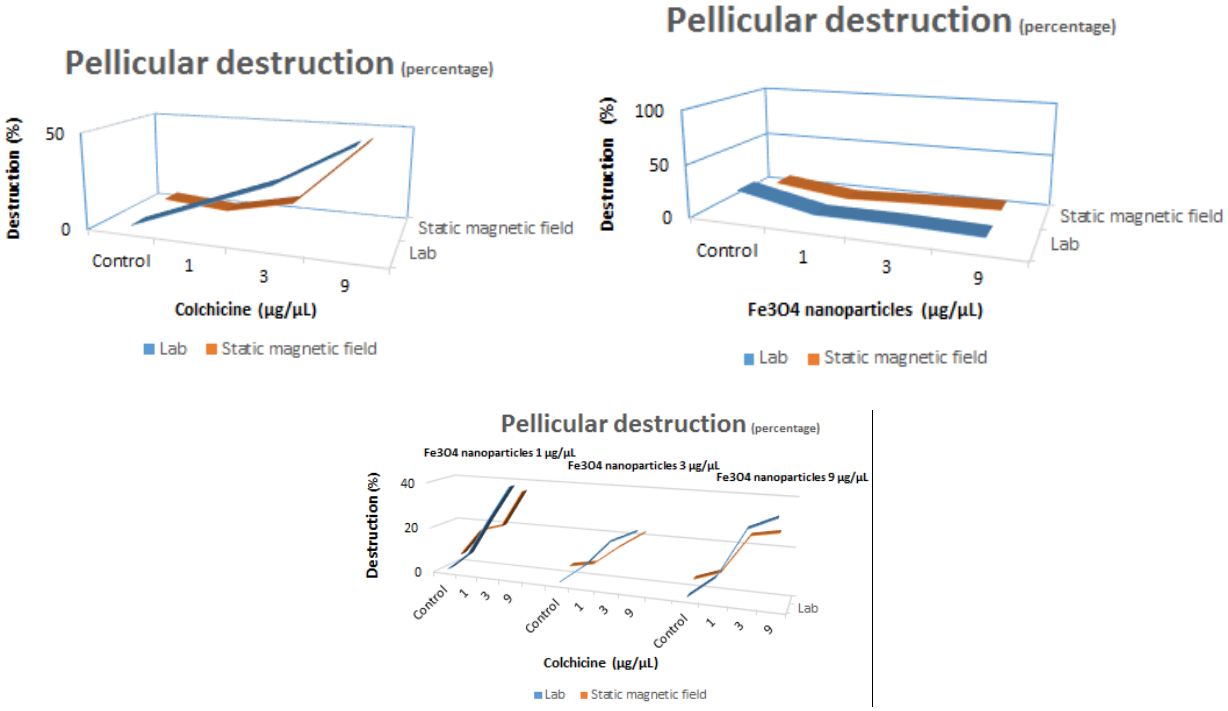

Fig. 7. Investigating the structure of pellicle with methyl fast dye. Single cells were stained with methyl fast after fixation and the images were quantitatively compared in the areas of 100 µm (in percentage). Destruction of pellicle structure in animals treated with colchicine is evident in the upper left figure. The upper right figure is for Fe3O4 NPs group. A little protection was shown with accumulative nano magnetite+ colchicine treatment (bottom).

3-8. Macro nuclear structure of *P. caudatum* after colchicine or/and nano magnetite exposure in normal and field samples

This effect is shown in Figure 8. The bean-shaped macronuclear structure of the cells was examined with hematoxylin staining. After the injection of nanomaterials, no destructive effects were observed in this structure. In cumulative use of colchicine and nano material, the degradation effect was partially improved (Figure 8 & figure 9).

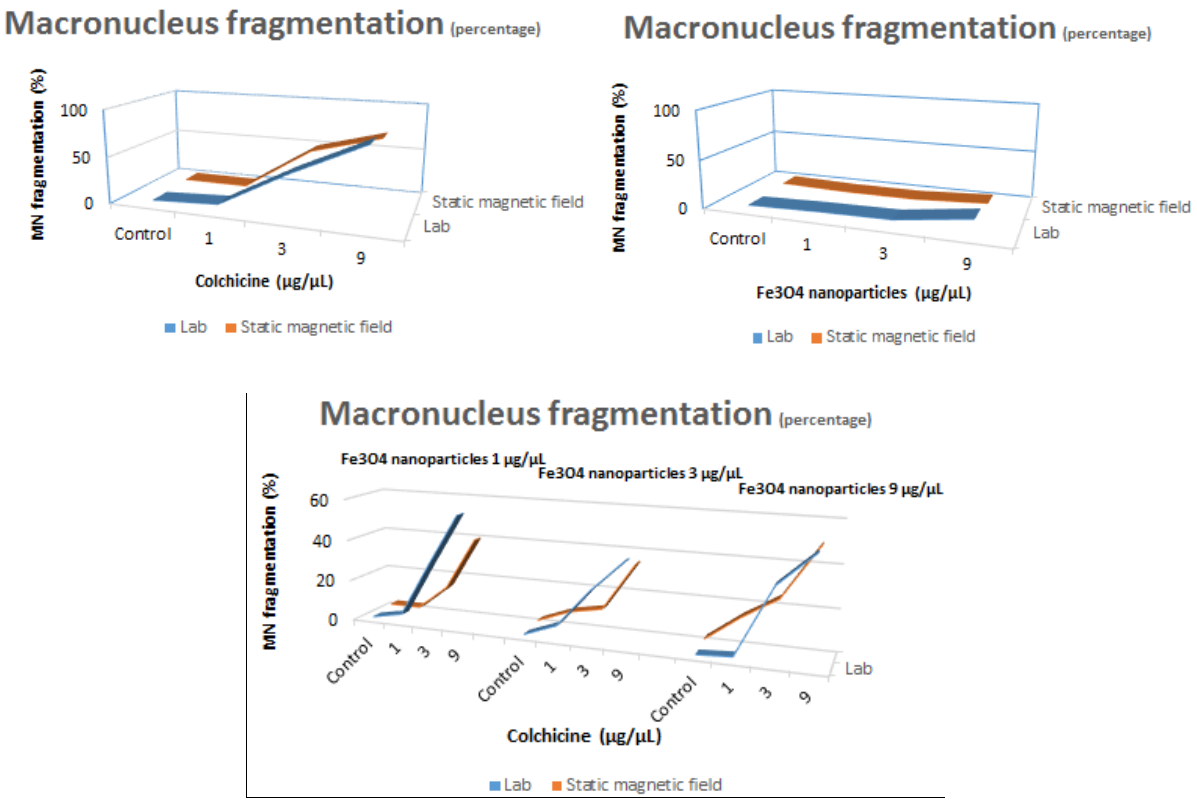

Fig. 8. The nuclear structure of *Paramecium*. The bean-shaped macronucleus was examined with hematoxylin staining and images were quantitatively compared in the areas of 100 µm (in percentage). After the injection of nanomaterials, no significant destructive effects were observed. In cumulative use of colchicine and nano material, the degradation effect was partially improved.

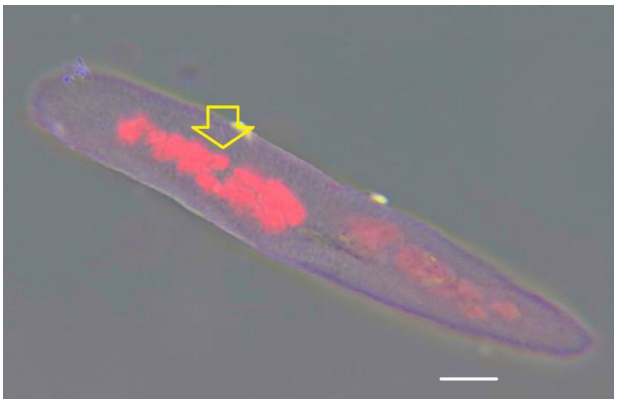

Fig. 9. The fragmented (arrow) nuclear structure of *Paramecium* under hematoxylin staining. Scale bar is 50 µm.

3-9. Examination of *P. caudatum* beta tubulin with antibody

Animals from the control groups, colchicine alone, magnetite nanomaterials alone and colchicine + magnetite nanomaterials, were studied in a laboratory environment and under the influence of a SMF with beta-tubulin antibody (Figure 10).

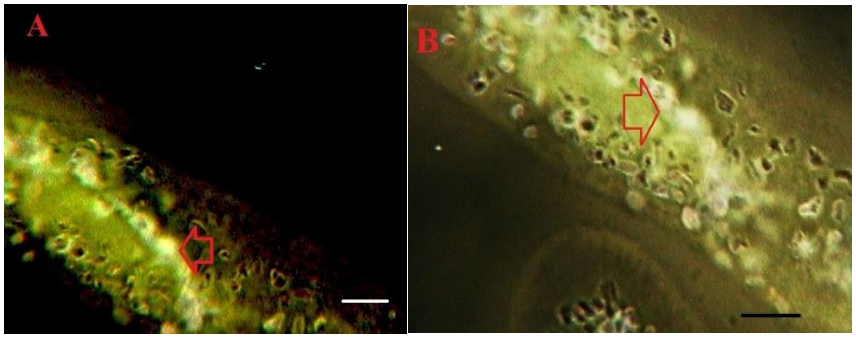

Fig. 10. Investigation of *Paramecium* beta tubulin with antibodies. A: normal group (laboratory) and B: field group. No change in tubulin intensity was observed in any case.

3-10. Nitric oxide activation test results

In this research, NADPH was used as an indicator for the activation of the NO production system, and nitrobutrazolium (NBT) played the role of cofactor. The results can be seen in Figure 11.

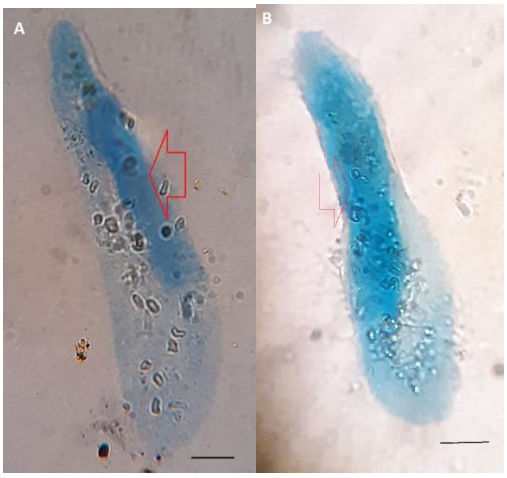

Fig. 11. NO synthase activation results. A: Normal group (laboratory) and B: Field group, no statistically significant change was observed in any of the groups (See positive blue reaction indicating NOS activation). Scale bar is 50 µm.

3-11. Incidence of escape response (avoidance) of *P. caudatum* when exposed to substances in the laboratory or under SMF

When a ciliate such as *Paramecium* faces some kind of obstacle or chemical stimulus, it reverses the direction of the cilia, it goes back a short distance and by relying on the back end, it deflects the front end; this state and reaction is called escape [1]. We studied exposure to colchicine and/or magnetite in Lab/SMF.

The number of escape movements during colchicine injection was dependent on colchicine doses, as can be seen in Figure 12. No significant effects appeared in the field. This is due to the relative reduction of swimming movement in the magnetic field because movement is an important component of this response. Nano material in presence of colchicine did not show significant protective effect.

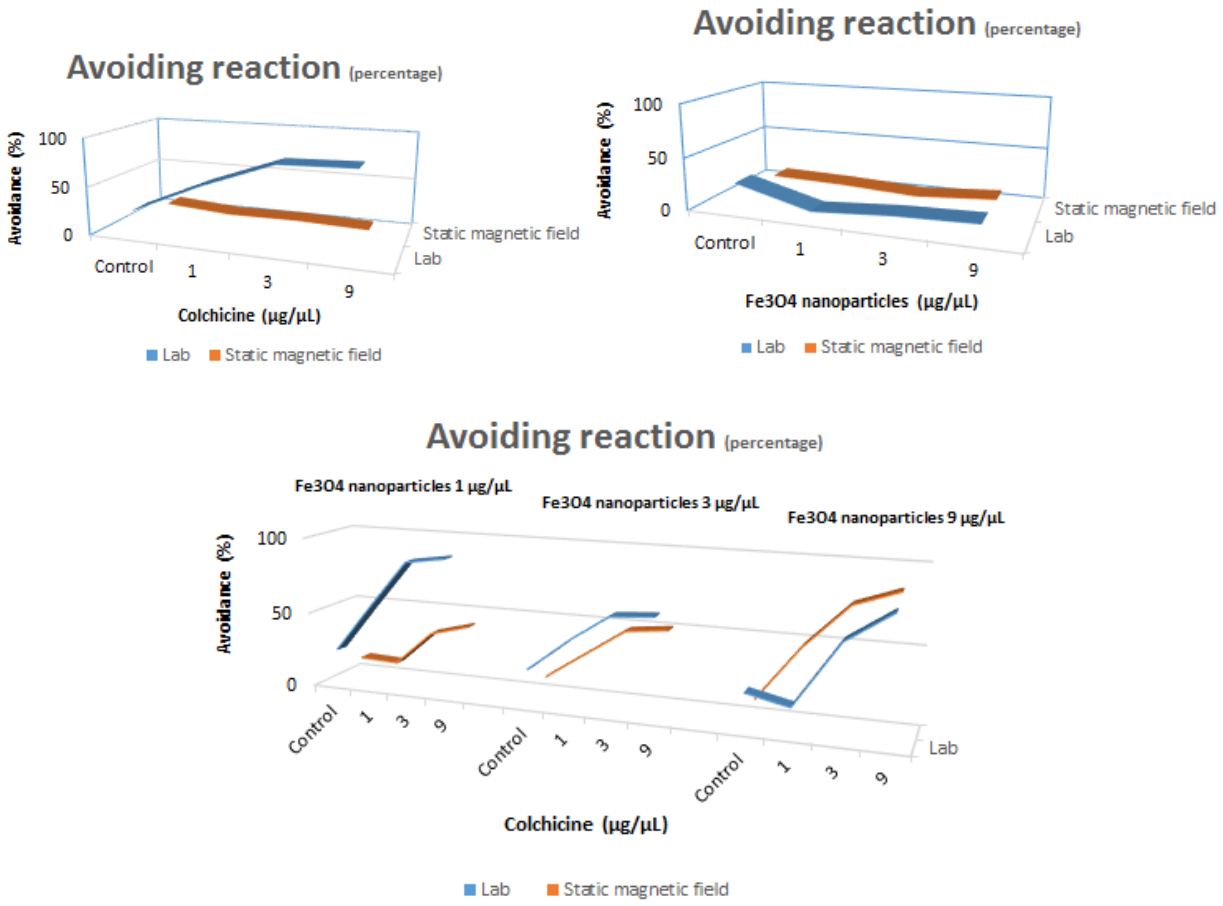

Fig. 12. Avoiding response of *Paramecium* exposed to colchicine and/or magnetite in Lab/SMF.

The number of escape movements during colchicine treatment increased depending on colchicine doses. There was no significant effect of nanomaterials, but we had only partial protection in escape under nanomaterials + colchicine.

4. Discussion

In this research, the effect of magnetic field and magnetite NPs on the toxicity caused by colchicine in *P. caudatum* was investigated. Animals that were under the influence of static magnetic field (SMF) or laboratory conditions were given colchicine alone or nano material alone or a combination of both. In all groups, movement and neuromotor system of animals were studied by silver nitrate staining. Then, for further investigation, the nucleus was stained with hematoxylin dye. Methyl fast dye was used to examine the pellicle. In order to study the tubulin of *Paramecium*, immunohistochemical method was used. Finally, the nitric oxide synthase (NOS) activation assay method was used to investigate the possibility of the oxidative role of NO.

The movement activities of *P. caudatum*, under the influence of colchicine, had a significant slowness, which indicates the toxic effect of colchicine.

Colchicine is an alkaloid that is used in the treatment of diseases such as gout, Mediterranean fever and cancer [2, 10]. By interfering with the organization of microtubules, especially mitotic spindles, colchicine can lead to the arrest of mitosis in the metaphase of dividing cells (both in plants and animals) [5]. Colchicine's c-ring analog is tropolone methyl ether, which can bind to the tubulin molecule and inhibit the polymerization of microtubules *in vitro*. Mescaline, which is an analog of the methoxy phenyl moiety in the A ring of colchicine, inhibits the assembly of microtubules. Therefore, the action of colchicine is dependent on these two rings, which are attached to microtubules and inhibit the movement of intracellular granules, thereby eliminating the secretion of various compounds outside the cell [5].

Colchicine disrupts the collective movement of microtubules by binding to tubulin. Colchicine binds to tubulin through reversible binding with high activation energy [11].

Since the movement of *Paramecium* is basically dependent on the correct functioning of microtubules, it was expected that by treating animals with colchicine, their movement activity would be disturbed, which was consistent with the results. The intensity of change in motor activity was dose-dependent, and the greatest change in motor activity was observed at the dose of 9 μg/μL. According to the obtained results, the samples that were affected by the magnetic field and NPs had less damage in motor activity. By colchicine in the presence of nanomaterial, a little protection was observed.

By staining the neuromotor system of *P. caudatum*, it was observed that the animals that were under the influence of colchicine suffered destruction in this structure, and of course, this destruction was consistent with the effects of colchicine on the swimming. In the animals that received the nanomaterial before colchicine injection, less destruction was observed in the neuromotor structure (a little protection). In addition, animals exposed to the magnetic field experienced less destruction after receiving doses of colchicine compared to the normal (Lab) group.

In agreement, in a study conducted in 2016 [6] researchers have found that colchicine slows down the movement of *P. caudatum*. It is possible that colchicine affects the calcium channels in the *Paramecium* membrane and changes the process of depolarization and hyperpolarization [12].

As mentioned, *P. caudatum* has two nuclei; the macro- nucleus and micro- nucleus. These nuclei are visible only if the cell is stained. The number of small nuclei is different in different species, and in a type of *Paramecium* (named

P. multimicronucleatum), it may reach seven numbers. In this model (*P. caudatum*), the number of micronucleus is one [1].

In an experiment conducted by Mikami in 1979 [13], the effect of colchicine on the *Paramecium* nucleus and its divisions was studied. The researcher has indicated that the macronucleus of *Paramecium* is fragmented under the influence of this substance.

In the present study, we also observed that colchicine causes macronucleus destruction in *P. caudatum* (fragmentation). This toxic drug turns the macronucleus from its normal state and causes its fragmentation. This destruction was increased dose-dependably. However, in the group that received only nanomaterial, no significant nuclear destruction was observed. Colchicine at 3 µg/µL, as a result of co-treatment with nanomaterial under magnetic field, caused less destruction in the nucleus of animals, which shows the therapeutic effect of magnetic field and magnetite nanomaterial lower doses.

In 2006, the effect of SMF on the movement of *Paramecium* was investigated by Guevorkian. [14]. He proposed that magnetic fields can be used as an invasive agent to manipulate the demography of unicellular organisms. In 2006, he and his colleagues found that the avoidance of *Paramecium* corresponded to SMF stronger than 3 T.

In another experiment conducted in 2006 by Elahee and his colleagues [15], they studied the effect of SMF on the growth of *P. caudatum*. The results of that experiment showed that the magnetic field reduces the growth of *Paramecium* with early population formation.

Rosen and his colleagues [16] tested the effect of a SMF of 0.126 T on the speed and movement pattern of *Paramecium*. According to their findings, when *Paramecium* is exposed to a magnetic field of 0.126 T, it experiences a significant decrease in movement speed and also confusion in the pattern of the swimming path. They have suggested that these findings can be explained based on the change in the function of specific ion channels in the cell membrane. In this research, due to the SMF alone, a slight decrease in the movement of *Paramecium* was observed. At the same time, the animals that were exposed to the magnetic field showed a less escape response when challenged with colchicine (this means that there is likely correlation between the induced slowing of the field and this response). Perhaps this difference in movement is due to the change in the concentration of calcium ions passing through the *Paramecium* membrane.

In this regard, Naitoh in 1968 [17] studied the role of calcium ion in the excitability and behavior of cilia. He has indicated that a small amount of depolarization in the cell membrane on the cathode side of the *Paramecium* and a minimal hyperpolarization in the membrane facing the anode are responsible for the response of the cilia, which leads to cathode orientation in the electric field.

But because tubulin is a substance in the structure of the neuromotor system (which is microtubular) and since the cytoskeleton network is also microtubular, it may have a main role in swimming movement. This matter was investigated and according to our observations, there was no significant change in the tubulin concentration.

But in relation to the escape reaction: it is worth mentioning that locomotor activity in ciliated single cells is controlled by the cell membrane through the physiological principles found in ligand-receptor situations. This is known as an escape reaction. When the front part of the ciliary membrane receives a mechanical stimulus or encounters a mechanical stimulus, it allows calcium to penetrate. This causes the formation of a depolarizing current in the receptor,

which spreads across the cell membrane by electrotonic diffusion. Depolarization causes a secondary transient increase in calcium that causes complete conduction across the cell membrane and a general calcium influx occurs. The result of increased intracellular calcium concentration is activation of a strong ciliary stroke for retrograde movement, which causes the organism to swim backwards. Moving forward or back to the first state is done by settling the intracellular calcium concentration in the cell cortex by means of diffusion. The control and coordination of movement in ciliates depends on several factors in addition to the excitable properties of the membrane. One of these features is the sensitivity of cilia to the concentration of intracellular calcium and other regulatory substances, the anatomical distribution of sensory receptors, especially in the cell membrane, and the conductivity properties of the cell, which allows the electrotonic release of a graded potential signal without the need for an all-or-none rule [18]. It is very probable that there is a connection between membrane electrophysiology and animal escape, but we have not recorded the membrane potential, which makes it difficult to have a definite opinion.

From the point of view of nanobiocompatibility, green bioproduction is a field of nanotechnology that provides a powerful, safe, stable, clean and environmentally friendly approach to produce NPs with a variety of applications in various fields of nanobiotechnology, medicine, and industry [19]. Iron oxide NPs (magnetite or Fe_3O_4) due to their unique quantum, mechanical, physical, optical, thermal, electrical, and superparamagnetic properties, have a wide range of applications in the field of nanobiotechnology and in magnetic resonance imaging, and etc [20-24].

No research was found on the effect of magnetite iron oxide NPs (Fe_3O_4) on *P. caudatum*, but it should be mentioned that in a research in 2021, the effect of hematite iron oxide NPs (Fe_2O_3 NPs) on the chemotaxis behavior of *Paramecium* was investigated. According to the results of that research, negative chemotaxis behavior was obtained when *Paramecium* exposed to Fe_2O_3 NPs (especially higher doses) and a decrease in the movement speed of *P. caudatum* under the inoculation of this nanomaterial have been reported. In high doses, the movement activity of the animal decreases significantly and the animal performs the avoidance reaction at a high speed when faced with the nanomaterial. Of course, the macronucleus changed very little. In other words, the fragmentation percentage of this nucleus was low [25]. In this study, accordingly, Fe_3O_4 NPs relatively decreased the speed of movement in *P. caudatum*.

NADPH was used as a marker indicating the activation of the NO production system in this study. As mentioned, NO contributes to vascular homeostasis by inhibiting vascular smooth muscle contraction, platelet aggregation, and leukocyte adhesion to the endothelium. Individuals with atherosclerosis, diabetes, or hypertension often show defects in NO pathways [9].

No significant activation of the NO system was seen in these treatments. This may need further investigation.

5. Conclusion

Although the magnetic field itself had little adverse effect on the movement of the animal (*Paramecium*), the animal's movement under colchicine exposure was improved in the presence of magnetite NPs. In the case of colchicine, this effect was consistently associated with the destruction of the macronucleus and pellicle, and most importantly, with the destruction of the neuromotor system. But surprisingly, there was no significant difference in cellular tubulin

density. The protective effect of nanomaterials was observed only very little and with lower doses, which shows the adverse effect of any exogenous material, including nanomaterials, in living organisms [26-30].

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Author contribution: M.K. proposed the research plan and designed the experiments. M.T. completed the research. A.H. provided the nanoparticles. M.K. and M.T. analyzed the data and prepared the paper. All authors reviewed and agreed the final manuscript prior to submission.

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