Nanomed Res J 9(4):411-423, Autumn 2024

RESEARCH ARTICLE

Calcium current block enhances the toxicity of CuO nanoparticles to motor and nuclear units of an *in vivo* single-cell model exposed to a static magnetic field

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ARTICLE INFO

Article History:
Received 09 Aug 2024
Accepted 26 Sep 2024
Published 01 Dec 2024

Keywords:
Paramecium caudatum
Static magnetic field
Magnesium sulfate
Copper oxide nanoparticle
Diamagnetic state

ABSTRACT

Objective(s): MgSO4, a calcium (Ca) channel blocker, can disrupt ionic homeostasis. However, its effect on the synergy of nanomaterials in static magnetic fields (SMF) with therapeutic use has not been investigated in an *in vivo* model. We used the blocker alone and with CuO nanoparticles (NPs) to investigate the synergy of SMF and NPs in living *P. caudatum*.

Methods: The experimental model was grown in straw and purified after serial cultivation. The pure environment was then divided into two groups. One group was exposed to SMF (0.061 mT) for 72 hours and the second group was exposed to natural, geostatic laboratory conditions. A sample of each group (0.1 mL) was treated with MgSO4 or/and CuO NPs (1, 3, 9 µg/µL) under a fixed objective lens (4x) for 25 times in 30 seconds with 5-second time interval. The control group received only 1 µL of distilled water. Sigmoid and avoidance movements of paramecia were counted. Trichocyst, macronucleus, pellicle, Ca channel and nitric oxide synthase (NOS) activation were studied. All data were analysed using ANOVA (α = 0.05).

Results: No changes in Ca channel density or NOS activation were observed due to exposure of paramecia to MgSO4 alone. However, the movement of paramecia using MgSO4 with high doses of CuO NPs under SMF was reduced, and the pellicular and macronuclear units were destroyed, indicating increased toxicity of CuO NPs.

Conclusions: MgSO4 can affect the diamagnetic state of CuO NPs under SMF, thereby intensifying the non-protective effects of nanomaterials.

How to cite this article
Momen H., Karami M., Hajnorouzi A. Calcium current block enhances the toxicity of CuO nanoparticles to motor and nuclear units of an in vivo single-cell model exposed to a static magnetic field. Nanomed Res J, 2024; 9(4): 411-423.
DOI: 10.22034/nmrj.2024.04.008

INTRODUCTION

Today, Nano science and Nanotechnology is considered as one of the most important research and development fields among modern sciences [1]. Apart from industry, nanobiotechnology studies are of special importance in biological sciences [2]. One of the special applications of nanoparticles (NPs) in biological networks is based on their disinfecting properties [3], and hence they are used in antimicrobial dressings and disinfectant sprays in healthcare centers.

But it should be noted that metal NPs (MNPs) with different sizes, especially in very small sizes, have the potential to cause cytotoxicity [4, 5].

Of course, these substances can lyse the bacterial membrane, but if they are used in the body of a living organism, for example in tumor therapeutics [6], they damage sensitive cells, especially blood cells, including red blood cells. Prolonged contact

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between cells and NPs is likely to result in loss of cell surface integrity properties [7, 8].

Due to the large surface-to-volume ratio, nanomaterials can easily absorb drugs and pass through the smallest capillaries due to their small size [9]. Size affects many of their properties, such as solubility and chemical reactivity, so some of them interact with biological molecules, which is studied in nanotoxicology [10]. Since NPs are not effectively phagocytosed by macrophages, they are poorly cleared in the body and can damage tissues by depositing in different parts [11].

Metal oxide NPs are also a good choice for drug delivery and as biological sensors due to the possibility of binding to different proteins in the protozoan's body [12]. But they can also be poisonous [13]. Inhalation of these compounds can cause chronic inflammation and produce oxygen free radicals. One of the most important nano metal oxides that has received much attention in recent years is copper oxide [14]. One of the most important MNPs is CuO NPs, which have unique properties. Available in either monovalent or divalent forms, the particles are semiconducting, dark in color, and insoluble in water. They dissolve slowly in alcohol and ammonia, and are soluble in dilute acids. CuO NPs are used not only in industry, but also in agriculture and medicine [15]. Nanotechnology is used in the prevention, diagnosis and treatment of cancer. Since classical treatments such as chemotherapy, radiation therapy, surgery, etc. may damage healthy tissues and reduce tumor size, there is a need for a more effective and less complicated alternative, which is the use of a magnetic field (MF) [16]. This approach is because NPs are composed of two main parts: core and shell. The core has a dominant quantum effect, usually containing magnetic elements such as nickel, iron, copper or cobalt, as well as their oxides. While the coating is responsible for stabilizing and protecting the core against the chemical effects of the environment. Coating plays an essential role in the location and mode of effect and can play a role in the orientation of NPs [17]. In the new chemotherapy method, with the precise guidance and penetration of magnetic NPs to the exact location of the tumor through the external magnetic field, more effective, faster and more targeted treatment can be achieved.

According to the above information, this research investigated the effect of the combination of static MF (SMF) and CuO NPs on the biological parameters of *P. caudatum*. This organism is a ciliated protozoan that, in addition to many basic principles of eukaryotic cells [18] is a very suitable alternative model of vertebrates based on ethical guidelines (replacement). Also in terms of cost and energy savings is preferred to laboratory mice and other small mammals. In this research, the neuromotor system and movement, nucleus and pellicle, calcium (Ca) channels using magnesium sulfate and protozoan behavior have been studied and new achievements have been made. This model simulates axoplasmic transport in a nerve by having a neuromotor system consisting of microtubule complexes. According to other sources, this single-celled organism is a moving neuron due to the microtubule structure of its neuromotor system, as microtubules are similarly the basic unit of axoplasmic currents in neurons [19]. Furthermore, since this small organism is unicellular, it carries out all its functions and physiology with this single cell and is therefore an excellent model for neurophysiology [20, 21]. We investigated the distinct effects of CuO NPs and a SMF on swimming and avoidance behaviors and nuclear and pellicle units of a well-known unicellular organism, *P. caudatum*. We studied how they affected the structure and function of the organism. We further investigated whether they showed synergistic impact under the application of a Ca channel blocker (MgSO4). We also studied the involvement of the nitric oxide (NO) system on the interactions.

MATERIAL & METHODS

In vivo study

In this study, *P. caudatum* was used, which is usually ovoid or egg-shaped, measuring 100 to 200 μm. It has a prominent oral groove and two contractile vacuoles. And thousands of trichocysts in its pellicle are visible under a light microscope. It has fast spiral (sigmoid) swimming and feeds mainly on bacteria and is found abundantly in sediments and debris contaminated with organic matter. We obtained this single-celled organism from the temporary ponds around our university campus and, after extensive subcultures (an average of 7 passages) with the help of straw-based nutrient media, provided a pure and rich culture environment for this micro-organism. In this research, all ethical guidelines were followed and ethical approval was obtained (IR.SHAHED.REC.1402.010.).

Materials

The straw was provided from the Faculty of Agriculture of Shahed University. CuO NPs were obtained from the Nanotechnology Laboratory of Shahed University. Methylene blue, and Methyl Green were obtained from F Arman Co., Tehran, Iran. NADPH, NBT (Merck, Germany), Antibody Kit (for Ca channel) were purchased from Santa Cruz Biotechnology, Santa Cruz, California, U.S.A (catalog No. sc-28828). Entellan glue was bought from Merck, Germany.

Sonoelectrochemistry for the production of CuO NPs

In the production process of CuO NPs used in this research, two aspects of ultrasonication were presented for the synthesis of divalent CuO nanostructures (NSs). In the first application of ultrasound, a copper tip was made for an ultrasonic probe transducer and used for electrolysis and ultrasound irradiation processes. This method is called direct sonoelectrochemistry and is compared to conventional electrochemistry. Divalent copper oxide NSs are obtained after baking for both direct sonoelectric-thermochemical and conventional electrochemical methods. In the second application of ultrasound, copper NSs were produced by ultrasound ablation method and then the heating process was performed for oxidation. The formation of copper oxide NSs is confirmed by X-ray diffraction, field emission electron microscope and transmission electron microscope. The results show that the direct sonoelectrochemical method produces 4.2 times more CuO (II) NSs than conventional electrochemistry. The direct sonoelectrochemical method is a very flexible method and parameters in electrochemical, ultrasound, and the relationship between them can play an important role in the synthesis process of NSs. One of the most important advantages of this method is its greenness, fastness and high purity of the produced NSs [22, 23].

Growth curve of P. caudatum

Based on experience and daily observations, it took about seven to ten days for the protozoan to reach stable logarithmic growth. After that, they grow slowly and their population decreases due to increased metabolism and waste production, so organisms must be passed before that. Of course, variables such as temperature, season and light were also effective in this case, for example, paramecia grew better in environments that were under indirect sunlight for a while.

Accordingly, they were subcultivated in fresh culture medium every seven to ten days, and within seven weeks, a pure culture medium enriched with *P. caudatum* was also prepared. It should be notified that when paramecia are exposed to evaporation of water or exogenous substances, their volume increases and their internal contents bulge out (known as blebbing) and then eventually rupture.

Behavioral studies (motility measurement) of P. caudatum

The action through which an organism actively communicates with its surrounding environment is called behavior, and in other words, every stimulus leads to reactive behavior. In natural habitats for this protozoan, stimuli can be mechanical, chemical, optical or thermal. In protozoa, receiving the stimulus and the appropriate response to it happens inside the cell itself, which may lead to movement towards the stimulus (positive taxis) or avoidance of the stimulus (negative taxis) [24]. Experiments have shown that many individual cells react to certain chemicals with this type of movement.

P. caudatum has several movement patterns that can be an important factor for studying its behavior. One of the movement patterns is the S-shaped (sigmoid) spiral movement [20]. Therefore, each complete spiral in this research is considered equivalent to one movement.

The sample (0.1 mL of rich culture) was placed on the surface of the slide with a Pasteur pipette and studied under a photomicroscope (4x) for 30 seconds with 5 second intervals (the first 5 seconds are also for adapting to the environment).

These samples were studied as samples of the laboratory (Lab) environment (with normal geomagnetic conditions) and exposed (72 hours) to SMF and included both nanomaterial dose-groups and dose groups of single magnesium sulfate groups and also cumulative magnesium sulfate-nanomaterial groups. It should be noted that each sample had many repetitions (25 times). The average obtained in each sample for each dose group from both experimental groups (Lab or SMF) had high reliability and statistical value.

Staining

Several color reagents were used successfully on *P. caudatum*. Each time a sample was studied on the surface of the slide (from a Lab stock or field stock)

and for each dose, the slide was allowed to fix. Then the process of staining was done according to the following steps:

1) Hydration: use of reduced degrees of alcohol from 96%, and 70% to 50% (average 15 seconds each).

2) Specific staining: using a specific aqueous dye in low concentrations (0.1%).

3) Washing with distilled water. In some staining, detergents were also used (Triton-X-100).

4) Dehydration: using increasing degrees of alcohol from 50% to 96%. Each slide should be placed in containers containing alcohol for an average of 15 seconds.

5) Clearing: using two xylene (each slide for an average of 15 to 20 seconds each).

6) Mounting: using Entellan glue, and then covering with coverslip. The slide was dried for 24 hours and then studied by photomicroscope (Olympus, Japan).

Types of coloring

Methylene blue

With the chemical formula C16H18ClN3S is a cationic dye. It colors the nucleus of the cells dark blue or purple.

Methyl green

With the chemical formula C26H33Cl2N3 is a very suitable color for coloring the nucleus of many ciliates.

Immunohistochemical staining of Ca channel

Using monoclonal antibody, membrane voltage Ca channels were detected based on the kit catalog and previous experiments [25, 26]. These L type which found in cilia are of type Ca_v1, which are found in neurons and heart muscle [26].

Data analysis

All data were analyzed with SPSS software (version 22). Each experiment was repeated 25 times in order to increase the statistical range and reduce the standard deviation. After the normality test, analysis of variance (ANOVA) was conducted. Tukey's *post hoc* test was used to compare between groups. The alpha coefficient was 0.05. Images were analyzed with Image J, free Java.

FINDINGS

Species Identification

Specific keys used for identification (Figure 1): a long caudal tuft of cilia, two types of nuclei with a specific shape (macronucleus and micronucleus).

pH Optimzation

The pH of the culture medium containing paramecia was determined once a day for two weeks. After the optimal period (see growth curve), changes were made in this critical parameter. They had the best nutritional and growth conditions at a pH of about seven. With the change (acidic or alkaline), a decrease in nutrition and growth and reproduction of paramecia was observed (Figure 2).

Growth curve

First, a drop of 0.1 mL of the rich culture medium was poured on the slide using a pipette, and then we observed 10 views (shines) randomly with a light microscope lens (4x). The experiment was repeated many times and then the average count was presented (Figure 3). As can be seen from the graph, after an optimal period (preferably 7 days), the number of paramecia decreases.

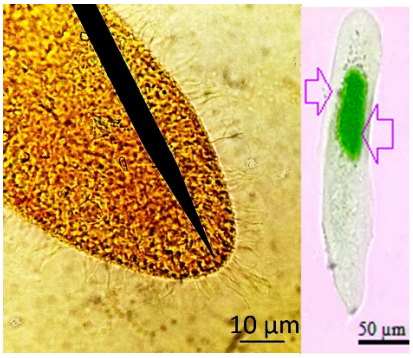

Fig. 1. Long caudal tuft of cilia, and two types of nuclei. Scale bar is shown in each image (10 µm & 50 µm).

Fig. 2. A trophic *P. caudatum*. The arrow points to the oral groove. Scale bar is 50 µm.

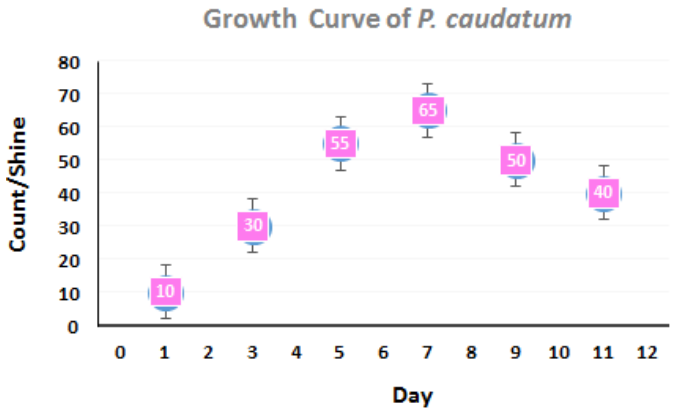

Fig. 3. Growth is shown as the mean count/shine over two weeks. A view under the 4x lens of a light microscope shows approximately 1/66.66 mL of sample volume. Therefore, by multiplying the average count by 66.66, the total number of cells in one mL of culture medium can be obtained.

Substances' effects

The effect of magnesium sulfate alone, CuO NPs alone and synergistically (MgSO4 and CuO) on Paramecium movement activity

Reduction of movements in high dose of MgSO4 was the most obvious effect on *Paramecium* (Figure 4). As shown in the figure, the reduction of mobility in Lab (MgSO4) is dose-dependent. The experimental groups were compared with the corresponding control groups. There is a difference between the Lab control group and the field control group, and this also shows the effect of reducing movement caused by SMF.

The macro-nucleus fragmentation, avoidance reaction, pellicle destruction and blebbing in Lab and SMF groups

Macronucleus Fragmentation

The destruction of protozoan's macro-nucleus at high dose CuO NPs was achieved in presence of a SMF, which is called fragmentation (Figures 5). The figure shows degradation of the macronucleus by a high dose of CuO NPs in the presence of a SMF.

Avoiding behavior

Paramecia avoidance was not observed following exposure (Lab and SMF) to magnesium sulfate or CuO NPs at lower doses (1-3 µg/µL). However, it was observed at a dose of 9 µg/µL (Figure 6). A small percentage of paramecia (5%) showed avoidance.

Pellicle destruction

Paramecia in the laboratory environment and under SMF in the presence of MgSO4 did not show the destruction except at the dose of 9 µg/µL. It was similarly observed in groups treated with CuO NPs in Lab, but occurred at lower doses in SMF (Figure 7).

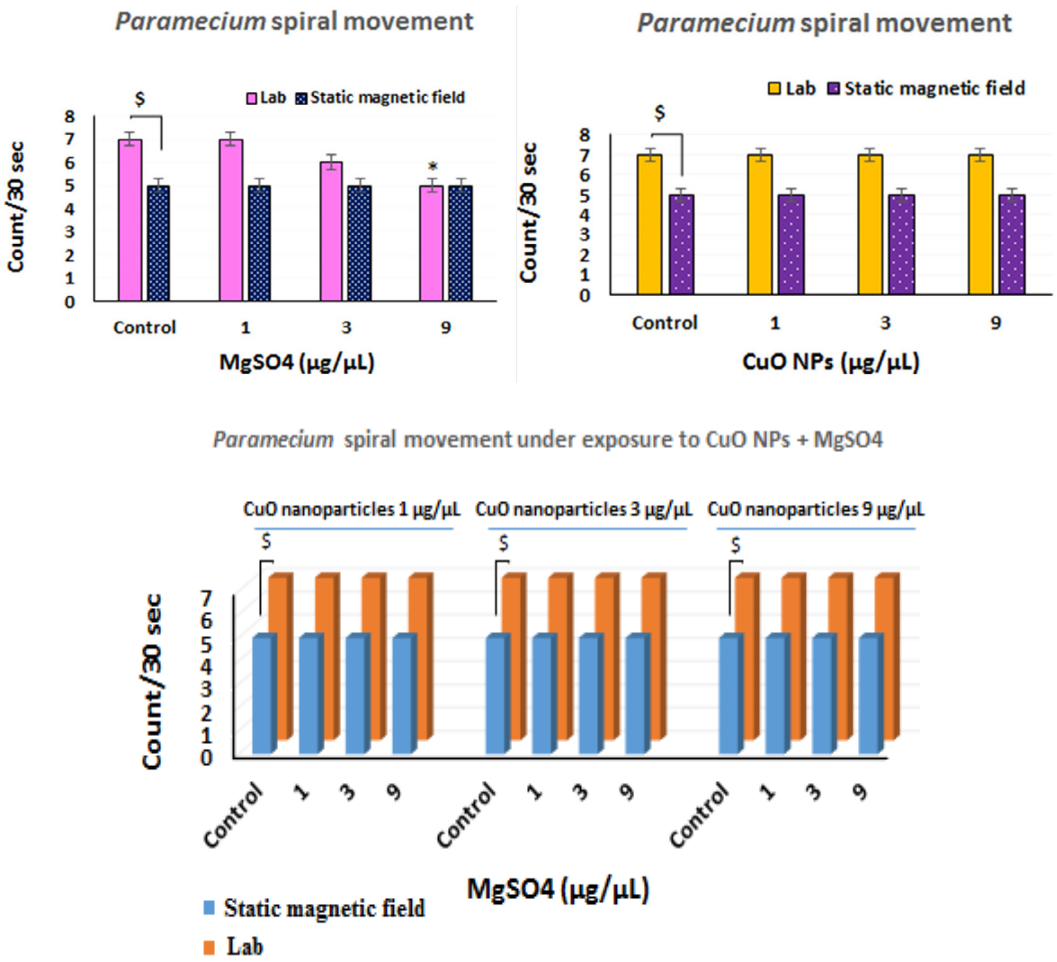

Fig. 4. The sigmoid (spiral) swimming movement of *Paramecium* was counted in the presence of MgSO4 or/and CuO NPs in Lab and under SMF conditions. Star is based on Tukey's *post hoc* test: *P<0.05.

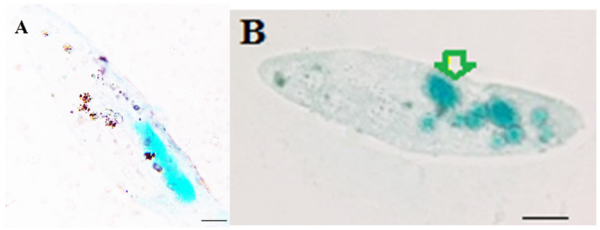

Fig. 5. Methyl green staining was used to demonstrate nuclear fragmentation. A: control, B: treated with CuO NPs 9 µg/µL. Scale bar shows 50 µm.

Blebbing

In a small percentage (5%) of paramecia (in Lab environment or under SMF) this phenomenon occurred at a dose of 9 µg/µL of magnesium sulfate (Figure 8 & Figure 9). But it was quite evident in the presence of a high dose of NPs. Blebbing was more obvious in CuO groups (see the image below showing the bubble).

Trichocyst discharge

CuO NPs at high doses under a SMF induced some discharge, which is considered as one of the protozoan's defense reactions (Figure 10).

Results of cumulative exposure to MgSO4-CuO NPs both in Lab and under SMF groups of paramecia

The results are shown in Figure 11. Paramecia

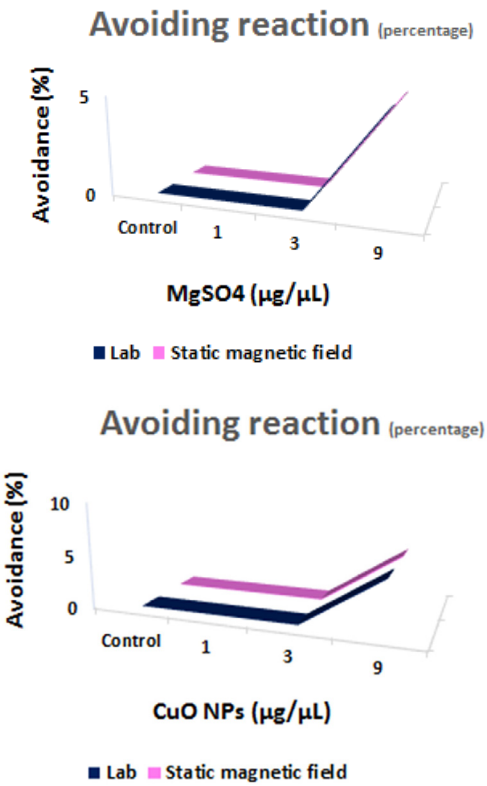

Fig. 6. Avoidance response of paramecia in the Lab and SMF when exposed to the substance at a dose of 9 µg/µL

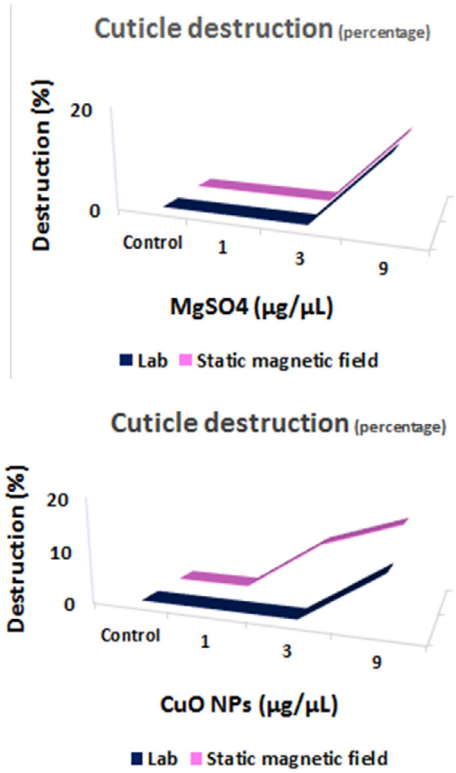

Fig. 7. Pellicle degradation in paramecia exposed to MgSO4 under the Lab and SMF.

Figure 8. *Paramecium* blebbing. The arrow shows the bubble.

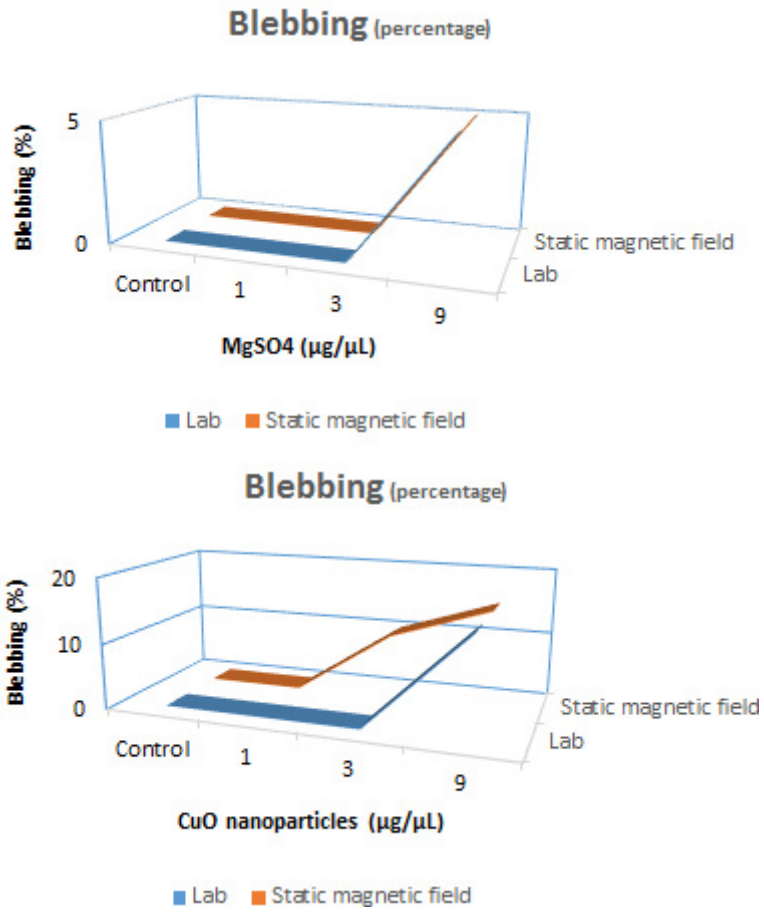

Fig. 9. Blebbing in paramecia exposed to MgSO4 or CuO under Lab/SMF.

were escaping in the Lab and the SMF when they were exposed to the substances in a synergistic treatment. The increase in that movement was significant with high dosage. Also, cuticle destruction was indicated both in Lab and SMF. It was observed by increasing the dose of magnesium sulfate (as a 5 second pre-injection) in the presence of CuO NPs. This behavior was more prominent in protozoans subjected to SMF. Bebbing response was also shown in Lab conditions and under SMF. It was observed with a dose-dependent increasing manner in both substances especially on micro-organisms that were in a SMF. And macro-nuclear fragmentation response of paramecia was observed in Lab and under SMF. Both MgSO4 and CuO NPs are effective at higher doses (magnesium sulfate was pre-administered 5 seconds) and can reinforce each other's effects with increasing doses.

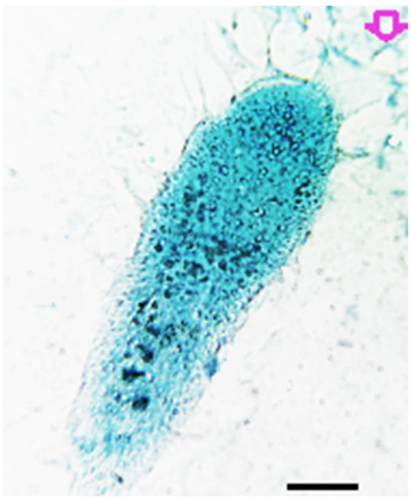

Fig. 10. Trichocyst discharge. Arrow indicates discharge. Scale bar is 50 µm.

Ca channel density and nitric oxide synthase (NOS) activation

The localizing point of these channels is at the level of the cilia in membrane of the protozoan's body, and no significant difference was observed in all groups (figure 12). There was no change in the NOS enzyme activation, which was investigated with the help of NADPH marker in *Paramecium*. NO is a very small, gaseous molecule with a key and important role in many processes and the NADPH-diaphorase assay is a specific biochemical marker for NOS activation (see 20-21). As can be seen in Figure 13, there is no difference to clarify.

DISCUSSION

In this research, the effect of magnesium sulfate, single CuO NPs and the combined effect of the above substances (magnesium sulfate as a pre-injection substance) on movements and a wide range of organs were investigated. Also the avoiding behavior and blebbing of *P. caudatum* in two environments (Lab and SMF) were studied. Based on our previous research, the effect of different doses of the mentioned substances was used to obtain reliable results. The movements of the protozoan in a period of 30 seconds in the normal environment (Lab conditions) and under the SMF in the face of different materials after the use of certain colors and experiments were analyzed. Statistical tests were performed and enabled us to discuss the results.

Behavioral study is considered as a widely used tool in ecotoxicology (environmental toxicology), which is of great importance and value in evaluating and tracking the toxicity of substances in unicellular organisms. Also, changes in movement can be an important and sensitive test to measure toxicity in single-celled organisms especially in ciliates like *Paramecium*, which have visible responses.

In this research, the NO system was investigated. NOS signaling system in living organisms is one of the effective parameters in oxidative stress [27]. Today, we know that NO is an unstable messenger molecule with a short half-life, which researchers consider as an organic molecule with high importance in thermoregulation and defense [28]. The activity of this molecule is highly concentration-dependent and involved in a wide range of physiological processes, but since NOS activation was not altered in the present results, we cannot clearly conclude a role for NO, so we prefer not to discuss.

We can mention the blebbing that we had in this study. According to many researchers, cells may disturb in the presence of various stimuli, including chemical stimuli such as poison. If the toxicity of the stimulus is such that the bleb is irreversible, a large volume of cytoplasm is released from the cell, leading to cell death. The researchers found that during blebbing, the hydrostatic pressure remains nearly constant, meaning that bubble formation is mainly driven by intracellular fluid flow rather than by water passing through the plasma membrane. It seems that Ca channels are involved in this phenomenon [29, 30]. The pellicular membrane of the *Paramecium* rises in the form of one to several

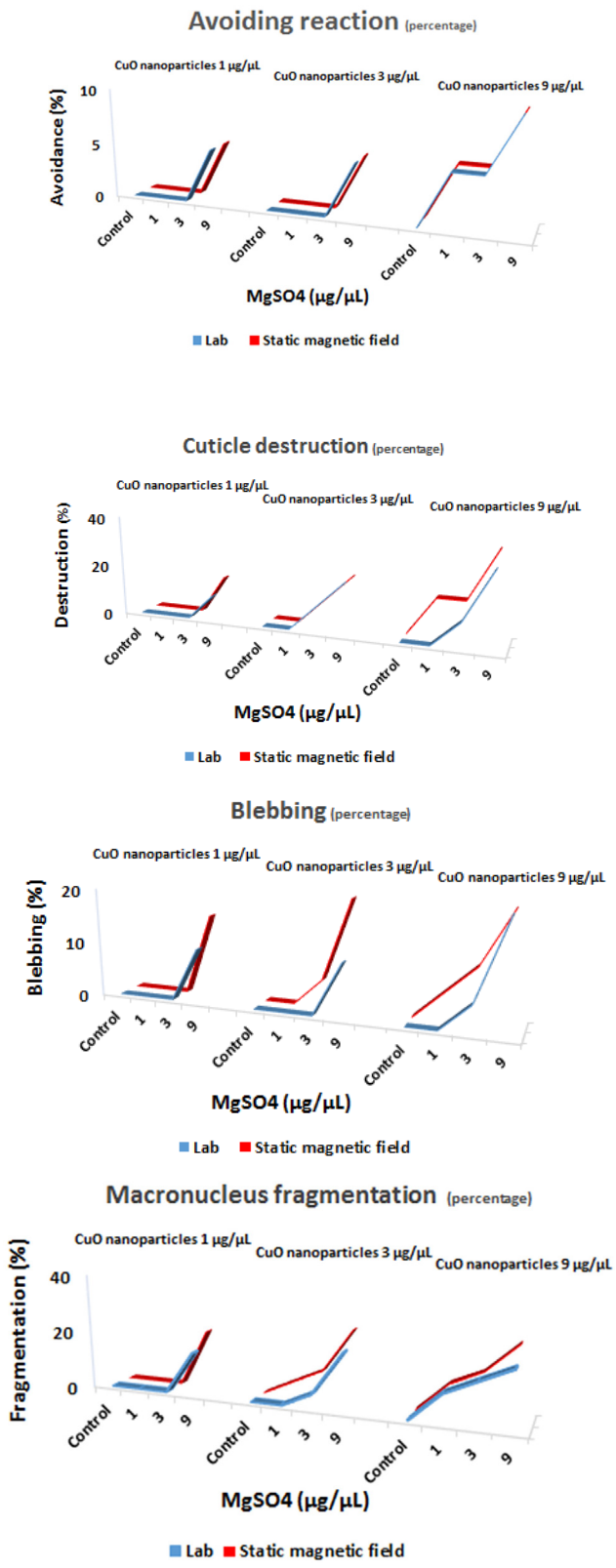

Fig. 11. Paramecia avoiding, cuticle destruction, blebbing response, and macro-nuclear fragmentation.

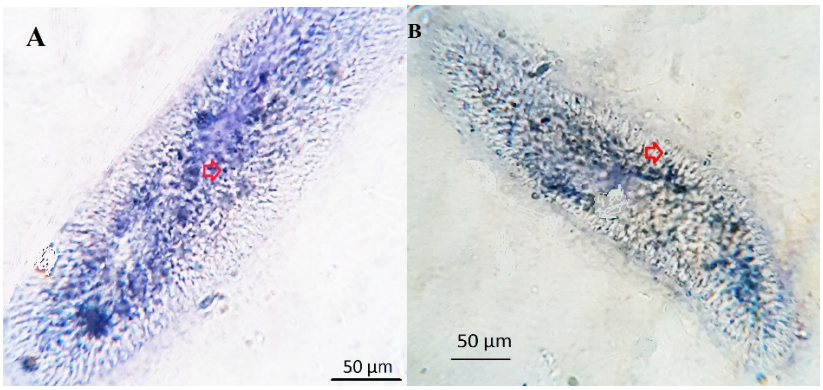

Fig. 12. Ca channel. The red arrow indicates the location of the channel with the help of its specific immunohistochemical diagnostic test. A: control membrane, B: subject group. Scale is shown.

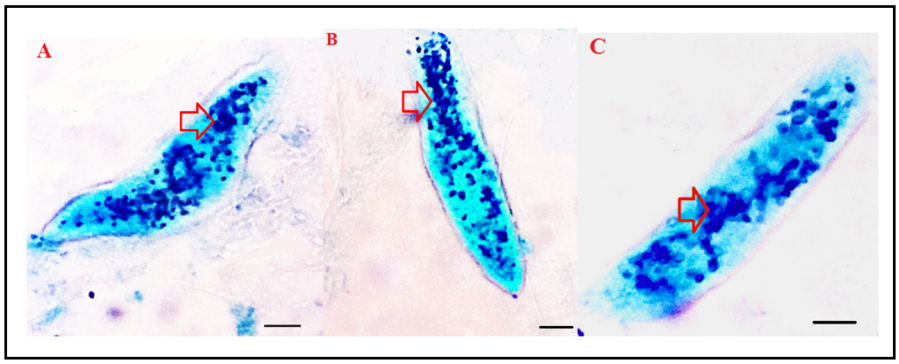

Fig. 13. NOS activation. The red arrow indicates the activation of the NOS in the control (A) and experimental groups, B: Lab, and C: SMF, respectively. Scale bar is 50 µm.

clear bubbles, which gradually fills with liquid and enlarges; this enlargement of the bubble continues until the membrane is separated from its basal membrane by a concentric bubble. Finally, this process leads to complete cell destruction. The process of blebbing is a common phenomenon during apoptosis (programmed cell death) [29].

The nanomaterials (NMs) used in this research induced bubbles that show the adverse effects caused by this type of exogenous material in living systems such as paramecia. Therefore, considering the unique properties of *Paramecium*, it should be explained that the study of this model provides an opportunity to investigate the relationship between the use of NMs and toxicity in living organisms. Therefore, it should be mentioned that this study provided a suitable cell model to investigate the toxic effects of NMs. It should be added that some *Paramecium* species are used as biometric indicators of pollution to evaluate the effects of toxic compounds such as drugs, pesticides, insecticides, etc [31]. In agreement, we have seen a decrease in the movement of paramecia. Decreased movement of living organisms is an example of the toxic effect of SMF and magnesium sulfate.

In this study, the density of Ca channel and probably the interference of this ion in the interactions with magnesium sulfate and NMs were studied.

The researchers have found that structural similarity or homology is seen in the subunits of Ca channels in *Paramecium* with the human IP_3 receptor or ryanodine [25]. Lodh and his colleagues [25] found that a group of receptors called Ca_v1 is found in mammals in addition to *Paramecium*, whose main function is to control Ca ion flow and Ca ion-dependent processes.

Knowing the information about the cellular-molecular mechanisms of magnesium sulfate, which is a Ca channel blocker, we tried to interfere with Ca channels and the effect of CuO NPs. And according to the current result, by blocking these

channels, the toxicity of CuO NPs increases.

Regarding CuO NPs, some studies have shown that NPs, especially MNPs, can reach the brain through intranasal route. These substances do not cross the blood-brain barrier, but they in the form of a spray enter the olfactory bulb and then through axonal transmission along the neuron, they can reach the brain. These NPs enter the systemic circulation and spread throughout the body. If NPs are taken orally, they will be absorbed through villi and intestinal epithelium [32]. Histopathological evaluation in a mouse model has shown that CuO NPs cause severe inflammatory changes in the lungs of mice at high doses and cause chronic inflammation or repeated induction of acute inflammation at low doses [33].

Magnetic NPs have more applications in biological systems. As the authors explain, they play a prominent role as contrast agents in producing high-resolution soft tissue images. Once these particles enter the cell, they are trapped by lysosomes, and such an escape strategy is an efficient way to reduce the toxic potential [34]. Although the cell's defense mechanism against the toxicity of high concentrations of NPs is not clearly understood. But, they may be trapped by phagocytosis and immune elements in the paramecia that need future study. In this research, we saw a decrease in the movement affected by the field in the SMF control compared to the Lab control, which we can attribute to the magnetic conditions. But the fact that CuO NPs did not induce more movement reduction under these conditions does not mean that it is non-toxic. Because we had many cases such as trichocytic discharge (defensive response) and macro-nuclear fragmentation and escape response, all of which contradict the non-toxicity of these substances.

CONCLUSION

A previous study has emphasized the regulatory role of SMF on T-type Ca channel [35]. In the present work, we observed that the use of a Ca ion blocker, MgSO4, could affect the swimming strategy of *P. caudatum* under SMF due to the effect on the diamagnetic state of CuO NPs, and, therefore, with this synergy between the Ca channel blocker and the NMs under the SMF, the non-protective effects of those materials are enhanced.

ACKNOWLEDGMENTS

We are grateful to the research vice-chancellor of Shahed University and *Neurophysiology research center of Shahed University* for supporting this research.

CONFLICT OF INTEREST

The authors of this article have no conflict of interest.

FUNDING

We did not receive any specific funding.

AVAILABILITY OF DATA

All data are included in this article.

AUTHOR CONTRIBUTION

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

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