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Research Paper

Synergistic Effects of Static Magnetic Field and Zinc Oxide Nanoparticles on *Paramecium caudatum* Cyclosis

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ABSTRACT

Background: Nanoparticles (NPs) have been increasingly noticed due to their unique properties. Static magnetic fields (SMFs) also have the potential to affect biological processes. We studied the effects of zinc oxide NPs (ZnO NPs) and SMF on cyclosis, motor activity, and dynein density in *Paramecium caudatum*.

Materials and Methods: Samples (0.1 mL) of *P. caudatum* pure culture were exposed to ZnO NPs (1, 5, and 25 µg/µL). Experiments were conducted under natural geomagnetic conditions (laboratory) and under SMF (0.061 T). Control groups received only 1 µL of distilled water. Over periods of 30 sec, the s-shaped movements, the acid/base status of cyclosis vacuoles, the escape behaviour, the levels of antioxidant factors and nitric oxide (NO), and dynein density were measured. Data were analyzed using analysis of variance (ANOVA).

Results: The field had a decreasing effect on the movement of *Paramecium* compared to the control. ZnO NPs, regardless of the conditions, caused an increase in movement at high concentrations. Avoidance was induced in exposure to high concentrations of NPs. The acid/base ratio characteristic of cyclosis vacuoles declined in high concentrations of NPs. Antioxidant factors, such as glutathione (GSH) and catalase (CAT) were reduced after exposure to NPs, but more severely, under the field. However, superoxide dismutase (SOD) and NO did not change significantly. Dynein density decreased after exposure to high concentrations of nanomaterial under the field.

Conclusion: ZnO NPs and SMF probably have synergistic effects on the physiology of *Paramecium*, including cyclosis and motility, by affecting the density of motor and scaffold proteins.

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Introduction

With the increasing development of nanotechnology in recent decades, the production and use of metal nanoparticles (MNPs), especially zinc oxide NPs (ZnO NPs), in various industries, including pharmaceuticals, cosmetics, and agriculture has increased significantly [1]. Due to their unique optical, catalytic, and antibacterial properties, these NPs have the potential to enter aquatic ecosystems. This fact has raised concerns about their biotoxicity to aquatic organisms [2]. On the other hand, living organisms are continuously exposed to magnetic fields (MF) of varying intensities, which can affect their cellular and physiological processes [3]. Studying the interactions between NPs and environmental physical factors, such as MF in single-cell organisms, such as *Paramecium caudatum* can expand the understanding of mechanisms of cytotoxicity and defence responses.

Paramecium is a free-living ciliated protozoan. Most paramecia can be easily cultured in the laboratory (Lab) and are ideal models for biological studies. Experiments on paramecia are recommended because of their biophysiological similarities to higher species. These tiny organisms meet their energy needs by feeding on other living micro-organisms, such as bacteria, yeasts, and algae. Food enters the unicellular organism through a structure called a cytostome and circulates through its body during the cyclosis. During cyclosis, the cytoplasm rotates continuously in a closed circuit [4, 5]. In *Paramecium*, during this phenomenon, the protoplasm rotates clockwise, and the vacuole formed at the cytostome moves in a circular path and its chemical nature changes. Finally, in terms of acidity and alkalinity, the substances contained in the cyclosis vacuole become digestible and absorbable [6]. This valuable organism is distributed in freshwater resources, including temporary water bodies, and plays an important role in the circulation of materials and energy in natural ecosystems. It is an important model for research on the food chain of aquatic ecosystems [4]. This interesting point prompted us to investigate the effect of ZnO NPs in this model.

ZnO NPs are well known and widely used due to their high photocatalytic activity and biocompatibility. They are used in sunscreens to protect against UV rays, cosmetics, antimicrobial skin ointments, plastics, and electronic semiconductors. Among MNPs, ZnO NPs are the third most produced NPs in the world, after nanosilica ($NanoSiO_2$) and nanotitanium ($NanoTiO_2$). However, the increased consumption of nanoproducts has also raised concerns and risks. These particles can be released into aquatic environments, posing potential toxic risks to aquatic organisms. They enter the zooplankton food chain and eventually end up in human food [5]. Studies have shown that static magnetic fields (SMFs) affect locomotion in paramecia. Also, according to the results of other researchers, their locomotion trajectory changes by affecting the diamagnetic anisotropic components of their body under SMFs [6]. This logic led us to investigate the simultaneous effect of ZnO NPs and SMF in this interesting model.

Materials and Methods

Subjects

P. caudatum was selected due to its rapid growth, high sensitivity to stress factors, and the possibility of direct observation of motility and vacuole changes. This organism was collected from temporary water sources around the main campus of Shahed University. This strain was identified based on three key features: A caudal bundle of cilia, two nuclei (1 macronucleus and 1 micronucleus), and distinct sigmoid motility, and was naturally cultured using alfalfa straw. Subsequent purification was performed by subculturing at 7–10 day intervals. The organisms were maintained at a constant laboratory temperature (about 25°C) and at a relatively stable pH during the culture period to ensure the health and normal activity of the cells. All ethical protocols were followed and the ethics code was granted by the local ethics committee.

Sampling

Each sample was 0.1 mL of a pure culture of *P. caudatum*, which was isolated and passaged for consecutive weeks (1 to 2 months). Experiments were performed in 25 replicates.

Nanoparticles

ZnO NPs were prepared at concentrations of 1, 5, and 25 µg/µL. For uniform dispersion, each solution was placed in an ultrasonic bath for 15 min before each experimental step. The particles were synthesized by a sonoelectrochemical method. All their properties have been previously published [7].

Groups

This study consisted of four groups: Control, ZnO 1 µg/µL, ZnO 5 µg/µL, and ZnO 25 µg/µL, which were studied under two environmental conditions (1- laboratory with natural geomagnetic conditions and 2- SMF). A SMF with a constant intensity of 0.061 T was applied to the samples for 72 h.

Biomarkers

Motor activity

The number of sigmoid movements in 30-second periods, with six intervals of 5 seconds, was counted under a microscope using a ×4 objective lens. This was repeated 25 times.

Avoidance behaviour

The percentage of sudden changes in direction and escape in response to external stimuli was recorded.

Food vacuole color change

Cells were stained with neutral red, and the type and percentage of vacuole color changes were examined to measure the acid-base ratio.

Molecular markers

Superoxide dismutase (SOD) and catalase (CAT) activities and reduced glutathione (GSH) and nitric oxide (NO) levels were determined in the samples. For this purpose, the samples were centrifuged and lysed using ultrasound waves to prepare cell lysates.

Dynein density

Dynein density was examined by immunohistochemistry.

Independent variables were the ZnO NPs concentration and the presence or absence of SMF. Dependent variables were movement, avoidance behaviour, vacuole color, and the levels of SOD, GSH, CAT, NO, and dynein density.

Dynein-specific immunohistochemistry

Fixed slides were first dehydrated and then placed in phosphate buffer solution (PBS) with pH=7.6 for 1–2 min. After that, the slides were placed (1–2 sec) in a very dilute solution of glacial acetic acid. Next, the samples were incubated with specific primary antibody (Ab) in a water bath at 36 ˚C for 60 min, and then washed four times with PBS. Then, avidin/biotin solution (for 10 min) and then chromogen (for 10 min) were added, and the slides were washed with PBS and then with distilled water. Dehydration was performed using increasing concentrations of alcohol (1 to 2 min each), followed by 2 min in xylene. Finally, the slides were mounted using Entellan (Merck, Germany). Given the sensitivity and specificity of this staining, all steps were performed with high precision.

Oxidative system agents

For cellular and molecular assays, an appropriate volume of pure culture of this unicellular organism was initially centrifuged and then subjected to ultrasonication to prepare cell lysates. All reagents, working standards, and samples were prepared according to the instructions provided in the kit.

Catalase assay

The CAT enzyme catalyzes the decomposition of hydrogen peroxide into water and oxygen and is capable of converting millions of H_2O_2 molecules into final products per second. The basis of the assay method is based on the peroxidative activity of the CAT enzyme present in the sample. In this reaction, CAT produces aldehyde (formaldehyde) in the presence of H_2O_2 and methanol (as a hydrogen donor). Subsequently, potassium hydroxide terminates the reaction and the resulting formaldehyde combines with the chromogen. The colored product formed can be measured spectroscopically at a wavelength of 540–550 nm.

Superoxide dismutase assay

One of the most important antioxidant mechanisms of the body against reactive oxygen species (ROS) is the presence and activity of SOD, a metalloprotein that catalyzes the conversion of superoxide anion to oxygen and hydrogen peroxide. Measuring this enzyme's activity using a standard kit is a simple, reproducible, and standardized method and can be performed in biological samples, such as cell lysates. The basis of this test is the inhibition of the autoxidation reaction of pyrogallol. Under normal conditions and in the presence of air, pyrogallol undergoes spontaneous oxidation and has a characteristic half-life. In this reaction, the addition of a sample containing SOD at an unknown concentration causes inhibition of pyrogallol autoxidation at a specific time. By comparing the results with the control, the concentration of SOD in the sample is determined.

Reduced glutathione assay

GSH is a key intracellular thiol tripeptide composed of glutamic acid, cysteine, and glycine. It acts as an antioxidant by protecting cells from free radical damage. It exists in cells in the reduced GSH and oxidized GSSG states. In healthy cells and tissues, more than 90% of to-

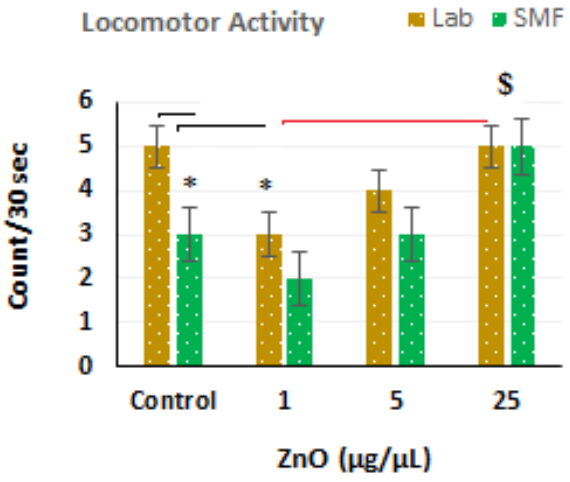


Figure 1. Sigmoid movements of *P. caudatum* (number of movements per 30 sec)

Note: Asterisk(s) indicate the difference from the control group at the 95% confidence level, and $ indicates the same confidence level for the difference between the maximum and low concentrations of the nanomaterial, as determined by Tukey's post hoc test.

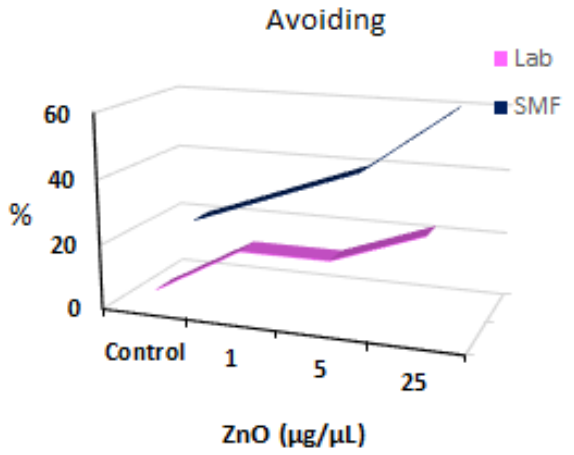


Figure 2. Avoiding behaviour

Note: The effect of high concentration is additive and unaffected by the environment.

tal GSH is in the reduced form, while less than 10% is in the GSSG disulfide form. High GSH concentrations are due to the highly active enzyme GSH reductase. An increase in GSSG relative to GSH indicates oxidative stress (OS).

Nitric oxide measurement

NO mediates many physiological processes, such as vasodilation, inflammation, thrombosis, immunity, and neurotransmission. It is synthesized from L-arginine, oxygen, and NADPH by the enzyme NO synthase (NOS) and is a highly diffusible molecule that exerts numerous effects. Various methods have been designed to measure NO in biological systems. One such method is based on the deionization reaction and spectrophotometric measurement. In this Griess reaction, the nitrite produced from the oxidation of NO is actually measured. The Natrix kit (Ontario, Canada) is based on the Griess reaction and provides a simple, reproducible, and standardized method for measuring NO in biological samples, such as cell lysates. NO is measured indirectly by reacting sulfanilic acid with nitrite in an acidic solution to form an azo dye, which can be measured at an optical absorbance of 520 to 590 nm.

Data analysis

All data were first checked for normality using the Kolmogorov–Smirnov test. Then, one-way/two-way analysis of variance (ANOVA) was used to examine the interaction between ZnO NPs and Lab/SMF. In cases of significant differences, Tukey's post hoc test was used to show differences between groups. The significance level in all analyses was $P<0.05$. Analyses were performed with SPSS software, version 22. Images were analyzed with Image J software (Java free, v1.41).

Results

Findings on movement and escape response

The effect of nanomaterials on the spiral and escape movements of *P. caudatum* over a 30-second period was assessed. All observations were made under the fixed objective lens (×4) of a light microscope for different concentrations of ZnO NPs under Lab and SMF conditions and compared to the control group (which received only 1 µL of distilled water) (Figures 1 and 2).

Cyclosis findings (acid/base ratio of cyclosis vacuoles)

The effect of the substance is dose-dependent and decreasing, and is unaffected by the environment (Figure 3).

Oxidative stress (OS) indices

Antioxidative factors, such as GSH and CAT were decreased under the field, and under nanomaterials the changes were more severe. However, SOD and NO did not change significantly (Figures 4 to 7).

Cyclosis indicators in vital staining

The rotation of the vacuole from the pharynx of the cell (the end of the oral groove) moves cyclically upward and then downward, and it finally empties at the anus

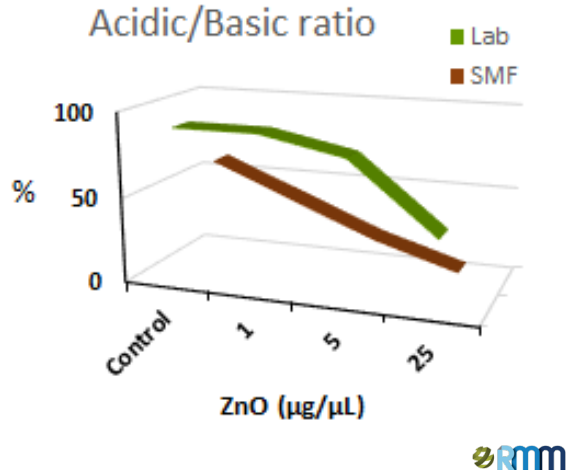


Figure 3. Acidic/basic ratio

Note: The effect of high concentration is reductive and has no effect on the environment.

of the cell (in the caudal third). Under vital staining with neutral red (0.001 g/100 mL), the color change of these vacuoles covers a pH range of 6.8 to 8, allowing assessment of the acid/base ratio. Microscopic images at 40× magnification are presented and shown in Figure 8.

Dynein density using immunohistochemistry

The intensity of the reaction in the control sample is high. In response to the high concentration of nanomaterial, this density is reduced, especially under the field (Figure 9).

Blebbing

This reaction is a type of defence response leading to death. A small percentage of cells exposed to high concentrations of ZnO NPs, especially in combination with the field, showed this reaction (Figure 10).

Discussion

In this research, the effect of ZnO NPs and a SMF on behavioral, physiological, and biochemical indices in *P. caudatum* was investigated and the following results were obtained:

• Locomotor activity is reduced under the field. ZnO NPs also have the same effect at concentrations of 1 and 5 µg/µL. This is compensated for at a concentration of 25 µg/µL.

• Volatility and even the acid/base ratio of cyclosis vacuoles show a relationship with the concentration of the nanomaterial, regardless of the environment.

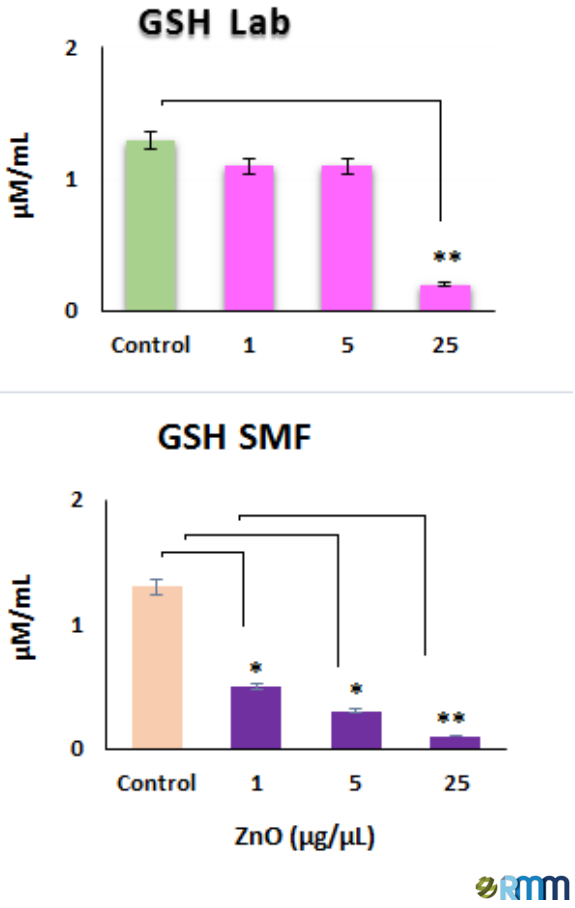


Figure 4. GSH levels

Note: Asterisk(s) indicate differences from the control group with 95% and 99% confidence, as determined by Tukey's post hoc test.

• Although the reduction of some antioxidant factors (GSH and CAT) helps explain these events, the best way to explain is to examine the density of dynein—that is, the reducing effect of high concentrations of the nanomaterial, especially in combination with the field, on the density of this important motor and scaffolding protein on which the cyclosis process depends.

Due to their unique physicochemical properties, such as high specific surface area, high reactivity, and ability to penetrate biological systems, MNPs can cause significant biological effects [1, 2]. On the other hand, MF can also affect cellular processes, ion transport, and metabolic pathways [8, 9]. Therefore, investigating the simultaneous effect of these two factors can be important for better understanding cellular responses in unicellular organisms.

The results of this study on the locomotor activity of paramecia presented a challenge. On the one hand, when comparing SMF conditions with Lab conditions, motil-

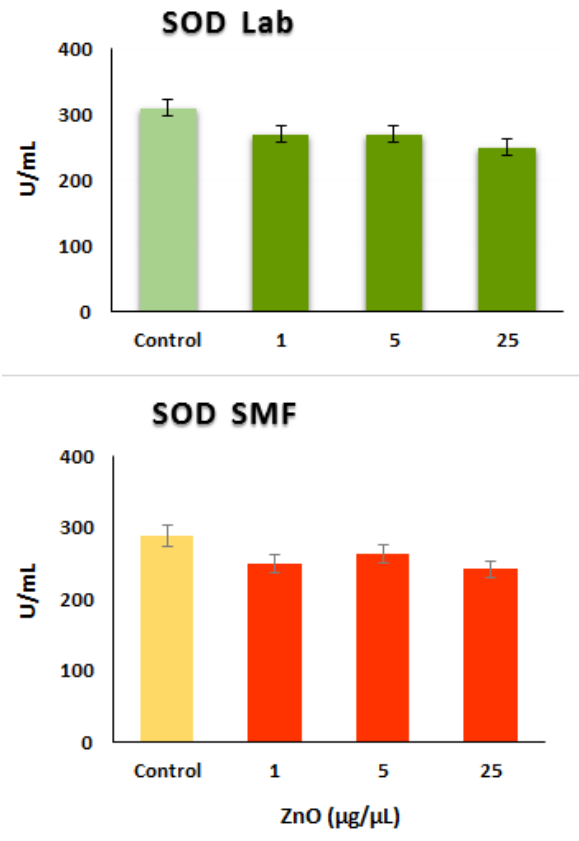


Figure 5. SOD levels

Note: No significant difference was observed.

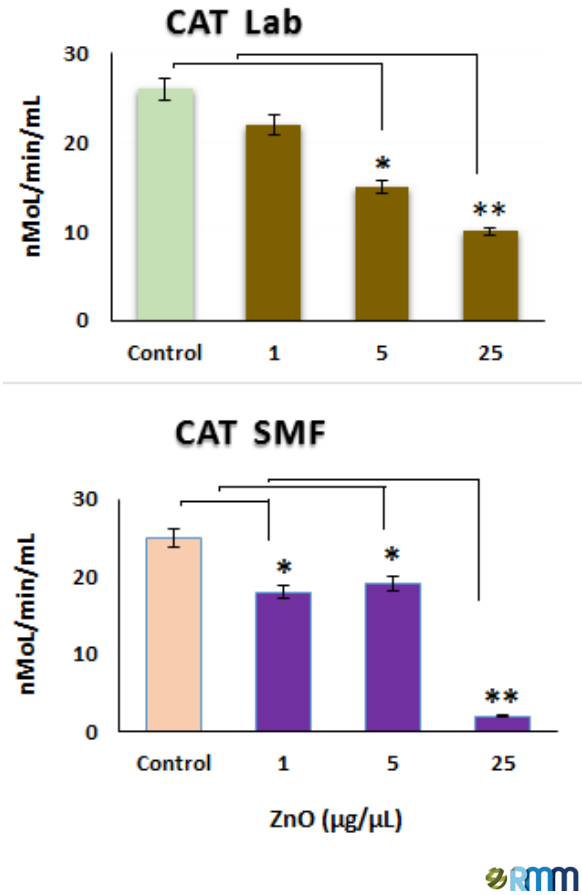


Figure 6. CAT levels

Note: Asterisk(s) indicate differences from the control group with 95% and 99% confidence, as determined by Tukey's post hoc test.

ity decreased, but it was compensated by increasing the concentration of NPs unrelated to the Lab or SMF.

By producing free radicals, MNPs can affect cellular structures, such as membranes, proteins, and cytoskeletal elements, and, as a result, cause a decrease in locomotion in unicellular organisms [1-3]. Therefore, the issue raised by the aforementioned reports can only be considered helpful for interpreting the decrease in locomotion under SMF.

It has been reported that MF can affect membrane potential and ion transport, and consequently change the motor behavior of cells [8, 9]. Therefore, it is likely that the effect of MF caused disruption in the function of the cilia and the motor system, as well as in the membrane potential of *Paramecium*, which unfortunately was not addressed in this study. This is one of the limitations of this study.

Avoidance behavior is one of the important defence responses in unicellular organisms against stressors. The present results showed that, in some treatments (with increasing nanomaterial concentration, regardless of the environment), the percentage of avoidance behavior increased. This could indicate the emergence of sensitivity in the neurophysiology of *Paramecium* to the stressor (high nanomaterial concentration) and the existing attempt to get away from the inappropriate environment.

Activation of calcium-gated ion channels in the membrane of *Paramecium* is one of the known mechanisms for the induction of avoidance responses. Some studies have shown that MNPs and HM can cause changes in ion flow and, consequently, a change in the direction of movement in *Paramecium* and other ciliates [10].

The increased occurrence of this behavior in the presence of a MF could also be due to the effect of the field on ion movement and changes in the electrophysiological activity of the cell membrane [9].

Regarding the vacuoles, Neutral Red is a vital dye used to examine the activity of lysosomes and cellular vacuoles. This dye accumulates in acidic environments and

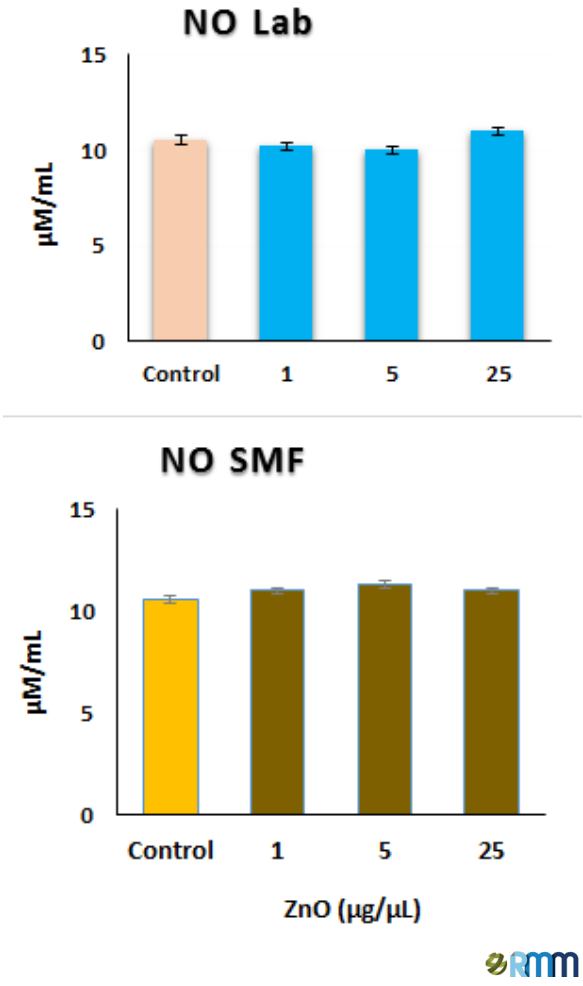


Figure 7. Mean NO levels.

Note: No significant difference was observed.

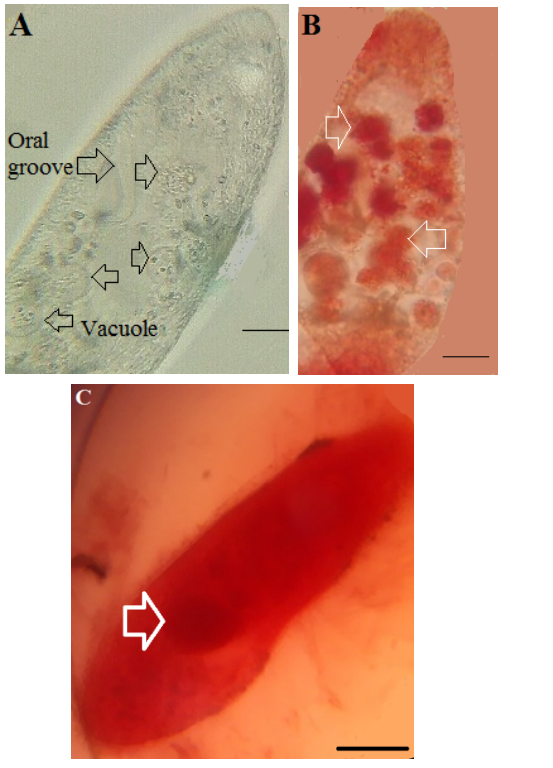


Figure 8. Cyclosis

A) Without dye interference, B) Under vital staining with neutral red to determine the acidity or alkalinity of cyclosis vacuoles, C) The death stage (cell nucleus stained). Scale bar is 50 microns

its color change can indicate a change in intracellular pH. In this study, with increasing concentrations of ZnO NPs, the acid/base ratio of vacuoles decreased. This phenomenon probably indicates a disturbance in the ionic balance and the function of submembrane organelles (vacuoles) of the cell. Various studies have shown that MNPs can cause damage to subcellular membranes, such as lysosomes, and change the acidity of these intracellular organelles [11]. The decrease in the above ratio in the presence of a MF could also be due to the effect of this field on ion transport processes and pH regulation in the cell [9].

SOD is one of the most important components of the antioxidant defence system in cells and plays an important role in neutralizing free radicals. In this study, a relative decrease in SOD activity was observed in treatments with ZnO NPs (relative to the control group), indicating an increase in OS in cells exposed to NPs. The decrease in the activity of this enzyme could be due to its excessive consumption in the process of neutralizing free radicals or its direct inhibition by ROS [4, 6].

Previous studies have also shown that exposure to MNPs can increase ROS production and consequently change the activity of antioxidant enzymes [1-4]. In the present study, the presence of a MF also caused a further relative decrease in SOD activity in some treatments, which is probably due to the increased production of free radicals under MF conditions.

CAT plays a key role in the decomposition of hydrogen peroxide and in protecting cells against oxidative damage [6]. The present results showed that CAT activity was significantly reduced due to ZnO NPs. This reduction is probably due to increased H_2O_2 production and OS on the cell defence system. Nanotoxicology studies have shown that many MNPs can disrupt the activity of antioxidant enzymes and disturb the oxidation-reduction balance in the cell [3-6]. The presence of a MF also significantly reduced the activity of this enzyme in this study, indicating a synergistic effect of the two factors.

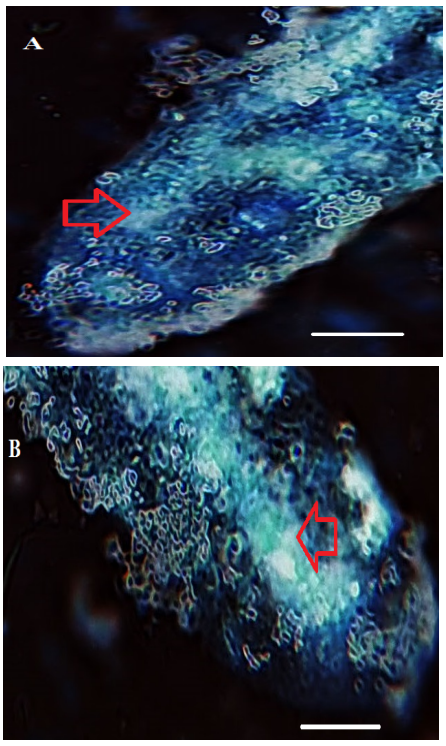


Figure 9. Dynein density with specific immunohistochemical staining

A) Control group, B) Combined exposure to a high concentration of nanomaterial and field

Note: Scale bar is 50 microns.

Our results showed that ZnO NPS are able to cause significant changes in the behavior, locomotor activity, and biochemical indices of *P. caudatum*. Also, a SMF can change cellular responses and in some cases, intensify the effects of NPs [3, 8, 9]. Decreased activity of antioxidant enzymes (SOD, GSH, and CAT) and changes in cell cyclosis vacuoles (based on staining with Neutral Red) indicate the occurrence of OS in cells [3, 6, 12]. However, the observed effects can be explained best by changes in dynein density. These findings are consistent with existing reports in the field of nanotoxicity and the effect of MF on biological systems [13-19].

It is well established in the literature that ZnO NPs exhibit paramagnetic behavior at room temperature due to the presence of structural defects, such as oxygen vacancies and zinc interstitials, which possess unpaired electrons [20, 21]. The paramagnetic nature of ZnO NPs has been confirmed through electron spin resonance (ESR) measurements, which show a characteristic signal at $g \approx 1.96$ corresponding to these defect sites [20]. The ZnO NPs used in our study are paramagnetic. The paramagnetic behavior of ZnO NPs has been shown ex-

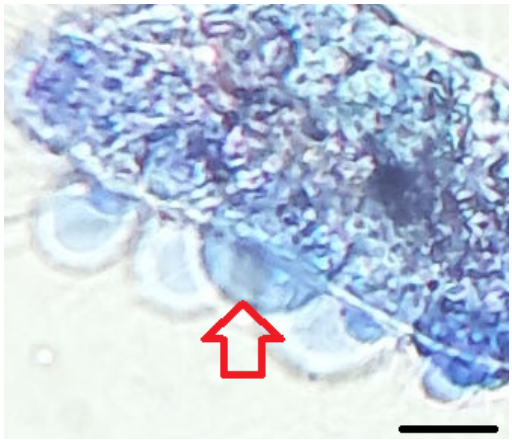


Figure 10. Blebbing

Note: The arrow indicates a swelling-shaped cytosolic protrusion. Scale bar is 50 microns.

perimentally; however, the SMF applied in our study was extremely weak. For a paramagnetic material, the induced magnetic moment is directly proportional to the field strength ($M = \chi H$, where χ is the magnetic susceptibility). At such a weak field, the magnetization induced in the ZnO NPs is negligible and insufficient to cause any significant physical movement, agglomeration, or direct magnetic interaction with the biological sample. The thermal kinetic energy (kT) at room temperature far exceeds the magnetic potential energy, meaning that the NPs undergo Brownian motion that completely overcomes any weak magnetic alignment tendency. Therefore, our study is based on the assumption that the NPs and the MF act independently without any direct mutual interaction. The weak MF strength used here is far below the threshold required to overcome kT and directly influence the NPs' behavior. Our research goal was to investigate the combined (or synergistic) effect of these two independent agents on the biological model (*P. caudatum*), assuming that the two factors do not directly affect each other but may produce a double or enhanced impact on the biological sample.

Based on the aforementioned information, ZnO NPs affect the vital parameters of unicellular living organisms, including the cytoplasmic streaming [22-24].

Conclusion

Based on the results of this study, it can be concluded that:

1. ZnO NPs have significant biological effects on *P. caudatum* and can cause behavioural changes (decreased

locomotor activity, increased avoidance behavior) and biochemical changes (disruption of antioxidant indices) in this unicellular organism. In support, it has previously been shown that NPs inhibit the growth of spring barley (*Hordeum sativum* L.) by modulating the degree of OS and the metabolism of antioxidant enzymes.

2. A SMF is able to affect some cellular parameters and, especially in combination with ZnO NPs, increase the intensity of OS responses. In a study on the modulating effects of ZnO NPs on OS in rats, the NPs at a low dose of 5 mg/kg had an ameliorating effect on the adverse effects of OS. However, the effectiveness of the NPs depended on the synthesis method, application time, and concentration of these materials.

3. The decrease in the acid/base ratio of cyclonic vacuoles in the treatments indicates that the combined stress of NPs and MF can lead to disruption of cellular homeostasis. In a study conducted on the effects of gold NPs, it was observed that these NPs reduce lysosomal activity by alkalizing the lysosomal lumen.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of Shahed University, Tehran, Iran (Code: IR.SHAHED.REC.1403.046).

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Authors contribution's

Conceptualization and study design: Manizheh Karami. Investigation: Samaneh Kanani. Formal analysis: Manizheh Karami and Samaneh Kanani. Writing the original draft: Samaneh Kanani. Review and editing: Manizheh Karami; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interests.

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