

Effect of zinc oxide nanoparticles on anxiety-related behaviors in male rats

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Abstract

The objective of this study was to investigate the influences of zinc oxide nanoparticles (ZONPs) on the anxiety-related behaviors of male albino rats. Because of the sexual differences found in the anxiety related disorders, thirty-six male albino rats 40-days old weighed from 130 to 150 g and divided into three groups; group I served as a control, groups II and III were subjected to daily intraperitoneal injection of ZONPs (30 mg/kg or 60 mg/kg, respectively) for 5 days sequentially. Each treatment contained four replicates of 3 rats each. Eight rats per treatment (i.e. 2 rats/replicate) were randomly taken and marked with green livestock spray for behavioral observation and anxiety testing on same day of injection. Elevated plus maize, open field, forced swimming, tail suspension tests were used for anxiety testing. Afterwards, the rats were sacrificed, and the histopathological evaluation of brain tissue was done. The results showed that the intraperitoneal injection of ZONPs reduced the standing behavior, climbing time of the forced swimming test, and increased the sleeping behavior, immobility time of the forced swimming test and agitation and immobility time of the tail suspension test ($P < 0.05$); while the ZONPs had no effects on alert activities, open field test, and elevated plus maize test ($P > 0.05$) in ZONPs injected groups. In addition, ZONPs induced necrosis of the nerve cells in cerebral cortex and the Purkinje cells in cerebellum.

Keywords: Rats, Anxiety, Zinc oxide, Behavior, Brain.

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Competing interest: The authors have declared that no competing interest exists.

Introduction

Nanotechnology creates and manufactures many types of engineered nanoparticles with size of up to 100 nm (Nanotechnology Research Directions, 1999). The small particle size of Zinc oxide nanoparticles (ZONPs) increases the surface area per unit mass and enhances the reactivity of them in a cell at very low concentration because of their special chemical and physical features and their elevated surface area to volume ratio (Drexler et al., 1987), so they are also being explored for their potential use in agriculture, material science, diagnostic and medical applications, food industry, and cosmetic (Siddiqi et al., 2018). ZONPs are being used in the food manufacture as additives and packaging due to their antimicrobial characteristics (Donaldson et al., 2005). Special interest being paid to their potential toxicity, including cytotoxic, proinflammatory and genotoxic effects due to increased use of ZONPs and exposure to these nanoparticles has been spreading out. ZONPs can enter the body through many ways such as injection, inhalation, and ingestion, they may then move to blood stream leading to harmful physiological effects.

Some reports consider that ZONPs as a substance of low toxic effect, due to presence of it in the human system as an essential trace element and its importance in the activity of many enzymes like alcohol dehydrogenase, carboxypeptidase, and carbonic anhydrase (Osmond and McCall, 2010; Zhao and Castranova, 2011). It is considered as an essential component or added as a nutritional supplement of many foods, so during evaluation of toxicity of nanoparticles in organs, zinc attracts little attention, however, many articles have revealed that increasing the dose of ZONPs can induce apoptosis in murine liver cells and enhance severe oxidative stress

(Sharma et al., 2012). Moreover, recent reports have revealed that ZONPs are toxic to rodents and animals, and they returned the ZONPs toxicity to the synthesis of metallic cations Zn^{2+} are the main causes of toxicity (Pujalté et al., 2011). Previous researches reported that some organ may show inflammation, oxidative stress and heart problems. Campbell et al., (2005) reported that ZONPs might mitigate cognitive impairment and abnormal behaviors in mice like anxiety-like behaviors, probably via modulation neuronal synaptic functions and plasticity (Xie et al., 2012). Moreover, ZONPs may organize ionic homeostasis and the biological functions of neurons and have great effect in central nervous system, which shed light on the possible application and treatment in neurotransmitter system disorders (Zhao et al., 2009).

Differences in dosage or ZONPs may be responsible for these conflicting results. Therefore, the objective of the study was to observe the influence of two dosages of ZONPs injected intraperitoneally on anxiety related behavior and brain histopathology in rat model. We hypothesized that intraperitoneal injection of high doses ZONPs would have a harmful effect on anxiety related

MATERIALS AND METHODS

I. Zinc oxide nanoparticles (ZONPs) preparation

ZONPs were synthesized using procedures published by Othman et al., (2017). Scanning electron microscopy (SEM) image of synthesized ZONPs was captured in The Electron Microscopy (EM) unit at Assiut University (Figure, 1). ZONPs suspension was performed using physiological saline solution. We dispersed ZONPs in the physiological saline solution (sterile and fresh), and ultrasonic liquid processor: Sonicator 4000 was used for 20

min to dissolve the suspension completely; ZONPs suspension was vortexed for 1 min before the intraperitoneal injections.

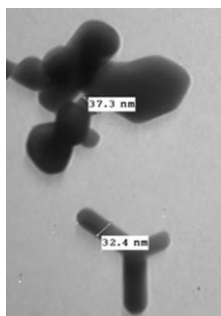


Fig. (1) Scanning electron microscopy (SEM) image of synthesized ZONPs.

II. Animals and housing

Thirty-six male albino rats 40-days old, weighing 130-150 gm at the beginning of the experiment and divided in to 3 groups with 4 replicates (3 rats per cage) (at 25°C, with free access to food and water under a 10:14 dark/ light cycle). Group I (control) injected with physiological saline solution, group II received ZONPs at dose of 30 mg/kg body weight, and group III received ZONPs at dose of 60 mg/kg body weight, through intraperitoneal injection for 5 days sequentially. Male albino rats were used in this experiment because of the sexual differences found in general anxiety disorders, many studies have also cleared that females are more likely to suffer from social anxiety disorders than males (Kessler et al, 1994; Schneier et al, 1992). Besides, observation of rat behavior revealed that aggression between dominant and subordinate males was negligible (van Zegeren, 1980). Further, it has long been known that, especially in animal ethology and neuroscience, using of rats have a number of advantages, such as the relatively large size of their brains, which makes brain surgery much easier, they are also much easier to handle than mice and less easily stressed by human contact (Meijer et al., 2007).

III. Behavioral observations

Twelve rats per treatment from four independent cages (3 rats per cage) were randomly taken and marked with green livestock spray for behavioral observation and anxiety testing. Observation of the rats' behaviors was done once a day from 10:00 to 12:00 am for 5 consecutive days, after injection of ZONPs in the same days of injection according to previously mentioned ethogram (Table 1) using instantaneous scan direct observation method (Altmann, 1974). The rats' behavioral patterns were recorded from outside of the cages with 1 m. The persons who conducted the behavioral observation were trained and they were blind to the treatments. Behavioral patterns of all rats per cage were scored 12 times per observation session (Mohammed et al., 2018).

Table (1) Behavioral ethogram.

Behavior	Definition
Sleep	The rat is motionless, either lying curled on its side, or sitting curled up, with its head tucked into the body.
Stand	The rat stands only on its leg.
Alert	The rat is sitting curled up, but the face is lifted, the rat either sits motionless, or appears to direct its head towards sounds outside the cage.

IV. Behavioral tests

Eight rats per treatment from four independent cages (2 rats per cage) were used in some behavioral tests to evaluate the anxiety-related behaviors (Figure, 2).

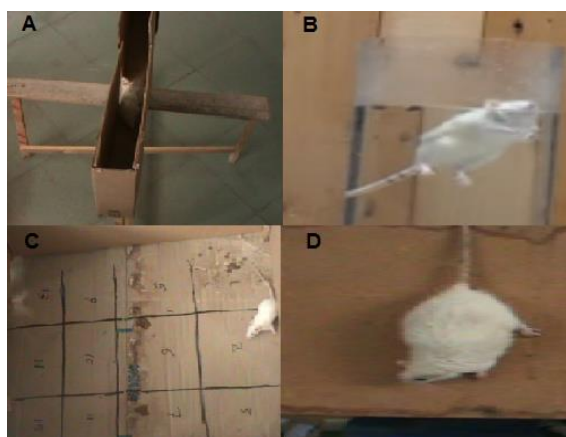


Fig. (3) Behavioral tests in the controls and ZONPs injected rats. (A) Elevated plus maze test. (B) Forced swimming test. (C) Open field test. (D) Tail suspension test.

1. Elevated plus maze test

Anxiety-related behaviors were assessed by using the elevated plus maze according to (Maaroui et al., 2009). The device was designed of yellowish wood. We elevated the device at height of 70 cm and the arms were 60 cm in long and 10 cm in width. We closed the close arms by 50 cm edges and left the open edges of the open arms to allow the rats to enter (Treit et al., 1993). We performed the test by putting the tested rats in the center of the device for 5 min. we recorded the time spent by the tested rats in the different arms of the device, in the same time, we calculated the number of enters in the different parts of the device. We used the alcohol to clean the device between the tested rats to eliminate the odor of the previous rat.

2. Tail suspension test

We used the tail suspension test accordingly to previously reported methodologies (Shinde et al., 2015). The tested rats were isolated and suspended 60 cm from the ground and fixed by adhesive tap 1 cm from the base of the tail for 5 min, we placed a thin wooden square under the

forepaws of the tested rat to decrease the weight on the tail by placing the forepaws on it. We recorded the immobility and agitation times. The immobility was defined as the disappearance of the movement of the rat, while the agitation was defined as struggling of the forepaws against the wooden square.

3. Open field test

Anxiety-related behavioral patterns (locomotion in the peripheral and central circle of the device, and the rearing) were investigated in the open field test according to (Ben-Slama et al., 2015). Rats in the control and ZONPs-treated groups were tested at the end of the experiment. The device was a wooden yellow circular enclosure (70 cm in high and 110 cm diameter). We divided the device in to central and peripheral circle; the central circle was divided in to 6 divisions of equal size. At the beginning of the behavioral test, the tested rats were put in the peripheral circle of the device. We cleaned the device using alcohol solution between the rats to eliminate the odors of the previous one.

4. Forced swimming test

We used the force swimming test to evaluate the anxiety-related behaviors accordingly to previously reported methodologies (Guzzetti et al., 2008). The test was performed by using a tank containing clean water (diameter 10 cm, height 15 cm). We put the tested rats individually in the tank for 5 min at the end of the experiment between 2:00 and 4:00 p.m. we placed a digital camera above the tank to record the behavioral patterns (immobility number, immobility time and climbing time). Before returning the tested rat to its home cage at the end of the test, we dried it by using a clean towel. Immobility was defined as the disappearance of the

movement of the rat, floating without any movement and stopping of the struggling behavior. Swimming was defined as the movement of the forepaws of the rat. Climbing was defined as the struggling of the forepaws of the rat against the walls of the tank.

V. Histopathological examination

At the end of the experiment, the rats were anesthetized by inhalation of overdose of pentobarbital followed by rapid cervical dislocation. This was followed by harvesting of brain tissues, which were placed in 10% formaldehyde. and brain specimens were cut and fixed in a10% neutral buffered formalin. The formalin-fixed samples were routinely processed, embedded in paraffin, and sectioned. Serial 5µm sections were stained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and eosin (Sigma, Missouri, USA). Afterwards, slides were examined microscopically, and the histological evaluation was performed in a blind fashion on coded samples, and a comparison was made with the sections from the control group.

VI. Statistical Analysis

The experimental design was conducted in a randomized block design. Pen was considered the experimental unit. Means of the data were analyzed by using the GLM procedure in JMP (SAS Institute, Cary, NC). The normality of the data was analyzed by the Shapiro-Wilk test. The overall effects of the ZONPs injection were analyzed statistically by one-way analysis of variance (ANOVA). Means were compared by Tukey- Kramer test when a significant difference was detected. Expression of the findings was designed as the mean \pm SE. Statistical significance was declared when the coefficients were at a probability of α less than 0.05.

Results

I. Behavioral patterns

The ZONPs effects on behavioral activities are presented in in Table 2. ZONPs injection increased ($P < 0.05$) rats' sleeping. Compared with control. Both doses of ZONPs increased the sleeping of rats. However, standing frequently was reduced. Moreover, there were no significant differences in alert behavior between the control and ZONPs injected groups ($P > 0.05$).

Table (2) Effect of ZONPs injection on some behavioral patterns in rats.

Treatment ¹	Behavioral pattern		
	Sleeping%	Standing%	Alert%
Group I	18 \pm 11 ^b	65 \pm 90 ^a	59 \pm 29
Group II	74 \pm 11 ^a	12 \pm 30 ^b	26 \pm 70
Group III	52 \pm 14 ^{ab}	14 \pm 11 ^b	23 \pm 10
P value	0.046	0.007	0.070

^{a,b}Means \pm SE with different superscripts in the same row differ significantly ($P < 0.05$).

¹Treatments were divided into three groups; group I served as a control, groups II and III were subjected to daily intraperitoneal injection of ZONPs for 5 days sequentially. Each treatment contained four replicates of 3 rats each.

II. Behavioral tests

1. Elevated plus maze test

The ZONPs injection effects on elevated plus maiz test parameters, time spent in open arms, number of enters in open arms, time spent in closed arms and number of enters in closed arms were presented in Table 3. There were no significant differences in all parameters of elevated plus maiz test between the control and ZONPs injected groups ($P > 0.05$).

Table (3) Effect of ZONPs injection on parameters of elevated plus maize test in rats.

Elevated plus maize test				
	Time spent in open arms(min)	Number of enters in open arms(min)	Time spent in closed arms (min)	Number of enters in closed arms (min)
Group I	0.86±0.44	0.10±0.10	2.05±0.20	0.25±0.14
Group II	---	---	2.19±0.22	0.39±0.22
Group III	---	---	2.47±0.01	0.11±0.11
P value	0.084	0.422	0.292	0.250

^{a,b}Means ± SE with different superscripts in the same row differ significantly ($P < 0.05$).

¹Treatments were divided into three groups; group I served as a control, groups II and III were subjected to daily intraperitoneal injection of ZONPs for 5 days sequentially. Each treatment contained four replicates of 3 rats each. --- means no rat entered these arms.

2. Tail suspension test

The ZONPs injection effects on tail suspension test parameters, immobility time, and agitation time were presented in Table 4. ZONPs injection significantly ($P < 0.05$) increased rats' immobility and agitation time in group III in comparison to the control.

Table (4) Effect of ZONPs injection on parameters of tail suspension test in rats.

Tail suspension test		
	Immobility time	Agitation time
Group I	1.83±0.07 ^b	1.47±0.05 ^b
Group II	1.96±0.02 ^{ab}	1.67±0.11 ^{ab}
Group III	2.05±0.003 ^a	2.08±0.11 ^a
P value	0.032	0.012

^{a,b}Means ± SE with different superscripts in the same row differ significantly ($P < 0.05$). ¹Treatments were divided into three groups; group I served as a control, groups II and III were subjected to daily intraperitoneal injection of ZONPs for 5 days sequentially. Each treatment contained four replicates of 3 rats each

3. Open field test

The means of the open field test parameters, rearing, locomotor activity in line crossing, locomotor activity in line central were presented in Table 5. The differences in rearing, locomotor activity in line crossing, and locomotor activity in line central were disappeared ($P > 0.05$).

Table (5) Effect of ZONPs injection on parameters of open field test in rats.

Open field test			
	Rearing(no.)	Locomotor activity in line crossing (no.)	Locomotor activity in line central (no.)
Group I	1.50±0.29	20±2.89	0.00±0.00
Group II	0.50±0.29	21±9.24	6.50±3.17
Group III	1.00±0.58	5±2.89	0.00±0.00
P value	0.30	0.175	0.073

^{a,b}Means ± SE with different superscripts in the same row differ significantly ($P < 0.05$).

¹Treatments were divided into three groups; group I served as a control, groups II and III were subjected to daily intraperitoneal injection of ZONPs for 5 days sequentially. Each treatment contained four replicates of 3 rats each.

4. Forced swimming test

The ZONPs injection effects on forced swimming test parameters, immobility time, immobility number, and climbing time were presented in Table 6. ZONPs injection significantly ($P < 0.05$) increased rats' immobility number and decreased the climbing time in group III in comparison to the control. However, there were no significant differences in immobility time between the control and ZONPs injected groups ($P > 0.05$).

Table (6) Effect of ZONPs injection on parameters of forced swimming test in rats.

Forced swimming test			
	Immobility time	Immobility Number	Climbing time
Group I	1.48±0.21	1.13±0.03 ^b	1.40±0.12 ^a
Group II	1.51±0.01	1.19±0.01 ^b	1.28±0.05 ^{ab}
Group III	1.53±0.06	1.34±0.2 ^a	1.06±0.03 ^b
P value	0.964	0.001	0.053

^{a,b}Means ± SE with different superscripts in the same row differ significantly ($P < 0.05$).

¹Treatments were divided into three groups; group I served as a control, groups II and III were subjected to daily intraperitoneal injection of ZONPs for 5 days sequentially. Each treatment contained four replicates of 3 rats each.

III. Histopathological findings

The brain sections of the control-treated rats showed normal cellularity with healthy neurons and prominent nuclei of the cerebral cortex (Fig. 3 A and B) as well as normal layers of the cerebellar cortex (Fig. 3 C and D). The neuropathological lesions observed in rats treated with 30 mg/kg or 60 mg/kg ZONPs were neuronal necrosis in the cerebral cortex (Fig. 3 E, F, I, and J). The necrotic neurons were red, angular, and shrunken and their nuclei were contracted and dense. In addition, necrosis of the Purkinje cells in the cerebellum were necrotic with shrunken cell bodies and condensed nuclei (Fig. 3 G, H, K, and L), which were prominent in rats treated with 60 mg/kg ZONPs.

Discussion

Nowadays, ZONPs could be dangerous due to toxicological and pathological effects. ZONPs retention in food and environment is common and exposure to them continuously may affect animal and human health (Bia et al., 2017).

Many researchers have revealed that ZONPs are toxic through different routes like oral or intraperitoneal injection (Dhawan et al., 2010). Plus, maize, open field test, tail suspension test and forced swim test are usually adopted to detect and evaluate depressive-like behaviors (Battagay et al., 1998). Here, our findings exhibited that ZONPs increased sleeping and decreased standing behaviors and increased the immobility and agitation of forced swimming and tail suspension tests, however, it decreased the climbing time of the forced swimming test.

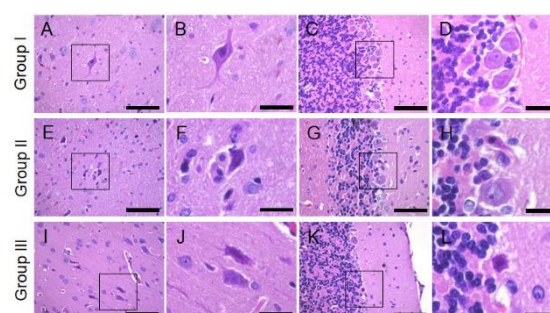


Fig. (3) Histopathological changes in the brain of control- and ZONPs -treated rats. (A-D) Brain sampled from control-treated group (group I) showed normal cellularity and nerve cells of the cerebral cortex (A and B) and normal layers of the cerebellum (C and D). (E-L) Brain sections from rats treated with 30 mg/kg (group II) (E-H) or 60 mg/kg (group III) (I-L) showed red and shrunken neurons with condensed nuclei indicating necrosis of the nerve cells in the cerebral cortex (E, F, I, and J) and Purkinje cells in the cerebellum (G, H, K, and L). Bars A, C, E, G, I, and K =200 μ m. Bars B, D, F, H, J, and L =50 μ m. Using of different bars to make the histopathological changes clearer.

In the current experiment, the results showed significant increase in the sleeping and decrease in standing. These behavioral changes may be related to depression when rats happened to be susceptible to despair and hopeless.

Our findings have shown that, intraperitoneal injection of ZONPs has no significant effect on the activity of rats measured in the open field and plus maze

tests such as locomotion, exploratory, and anxiety-like behaviors. These findings were supported by Amara et al. (2014) who revealed that, the absence of correlation between ZONPs accumulation in brain following ZONPs treatment and behavioral patterns of rats.

On the contrary, ZONPs treatment increased the immobility and agitation of forced swimming and tail suspension tests; however, it decreased the climbing time of the forced swimming test. Function of memory and cognition was conducted through the hippocampus, referring to the synaptic functions and neuron network, depending on the synaptic transmission characteristics and functions (Adolphs et al., 2005; Addis et al., 2007; Buckner and Carroll, 2007). Furthermore, ZONPs treatment induced neuronal necrosis in the cerebrum and cerebellum. Changes in the locomotion, memory, and anxiety-like behaviors, and emotion may indicate the depression and impairment of learning acquisition effectively. These behavioral and emotional changes could be attributed to necrosis of the brain cell (Min et al., 2009).

Conclusion

In the current study, the results indicated that ZONPs increased sleeping and decreased standing behaviors and increased the immobility and agitation of forced swimming and tail suspension tests. The current results supported that ZONPs may induce behavioral and emotional changes in rat model. However, further studies should be conducted to examine the mechanisms by which the ZONPs induce necrosis of brain cell.

Conflict of interest

We do not have conflicts of interest to disclose.

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