UDC 159.929:620.3(063)

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DIFFERENT EFFICACY OF NANOPARTICLE AND CONVENTIONAL ZnO IN AN ANIMAL MODEL OF ANXIETY

Received December 18, 2012.

As has been shown, trace element supplementation by zinc, e.g., in the form of zinc oxide (ZnO), can significantly influence the anxiety level. We investigated the effects of ZnO in the form of nanoparticles (NPs) in comparison with conventional ZnO (cZnO) in an animal model of anxiety. Adult male Wistar rats were divided into seven groups, control (receiving 0.9% saline) and six groups receiving 5, 10, and 20 mg/kg ZnO NPs and 5, 10, and 20 mg/kg cZnO. All drugs dispersed in 0.9% saline were injected i.p.; 30 min later, the anxiety level was estimated according to the results of the elevated plus maze test. ZnO NPs (5 mg/kg) and cZnO (10 and 20 mg/kg) significantly increased the normalized values of time spent in open arms (open arm time, OAT, %) in comparison with the control group ($P < 0.05$). This is indicative of the anxiolytic effects of these components; in addition, 20 mg/kg ZnO NPs reduced the intensity of locomotor activity ($P < 0.05$). The serum zinc concentration was increased manifold by anxiolytic doses of the components. All doses increased serum pH to 8.05-8.10 and kept this index constant for 24 h. These results indicate that the anxiolytic effect of ZnO NPs is much more intense than that of conventional ZnO, but the introduction of ZnO NP as a new drug for the treatment of anxiety disorders needs further investigations.

Keywords: anxiety, nanoZnO, plus maze, rats.

INTRODUCTION

Anxiety disorders, being a common mental health problem in the general population, affect up to 18% of individuals within a given time period and 25% of the individuals over their lifetime [1]. Generalized increases in the level of anxiety tend to precede depression and eventually develop into depression. Unfortunately, current drug therapies for psychological disorders are frequently not very successful, and many patients either do not respond to these treatments or suffer from side effects [2].

Zinc, an important prevalent trace element, is essential for both brain and systemic physiology. It has been found in a great number of protein structures, including important enzymes regulating a wide variety of cellular processes (e.g., cell division, DNA synthesis, etc.) and cell signaling pathways [3]. Zinc modulates functions of many receptors (including AMPA/kainate, NMDA, and GABA receptors) and of voltage-gated calcium channels [4-6].

During the past 50 years, it has been found that zinc deficiency in human populations is rather common, and nutritional deficiency of zinc affects many people in developing and developed countries [7]. A few studies showed that zinc deficiency induces anxiety-like behavior in animals. Also, studies on rodents (rats) suggest a causative role for zinc deficiency in the induction of depressive-like symptoms, reduced physical activity, anxiety, and anorexia [8]. Data presented by Whittle et al. [9] showed that dietary zinc deficiency in mice induces anxiety-related behavior in the novelty-suppressed feeding test and enhances the latencies of food consumption. Feeding with some zinc supplements, such as $ZnSO_4$, conventional ZnO (cZnO), and zinc methionine, was effective in reducing anxiety in rats [10]. For many years, feed manufactures prefer to use oxides of trace minerals (including zinc). Zinc oxide contains a greater concentration of this cation, as compared to sulfate, and cZnO is less toxic in comparison with other Zn compounds [11, 12].

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With the development of nanotechnology, the use of nanoparticles (NPs) to replace usual-scale particles has increased rapidly [13]. Manufactured NPs can be organic (polymers) or inorganic, such as nano powders of metal oxides and metal salts. Metal oxide NPs are the most frequently produced nanomaterials [14]. ZnO NPs constitute the extensively used engineered metal oxide nanomaterial; due to their unique optical, catalytic, semiconducting, piezoelectric, and magnetic properties, they are widely produced and technologically applied [15]. Recently, ZnO NPs have attracted the attention of biotechnologists, as they can be surface-functionalized with a wide range of metal and semiconductor core materials, thereby imparting useful properties with potentially wide-ranging therapeutic applications [16]. ZnO NPs can be ingested directly in food, used in food packaging, and as drug delivery agents. This ZnO form is used in the food industry and in biomedical applications due to its antimicrobial properties and also as anticancer drugs [17].

Some toxicological studies have, however, shown that when NPs enter into the human body through several distinct routes, including inhalation, ingestion, and dermal penetration, they can exert noticeable toxic effects at different levels of biological systems [18]. Therefore, a thorough investigation of the effects of this ZnO form on human health is necessary.

The aim of our study was to evaluate the effects of ZnO NPs and conventional ZnO in the plus maze test as an animal model of anxiety.

METHODS

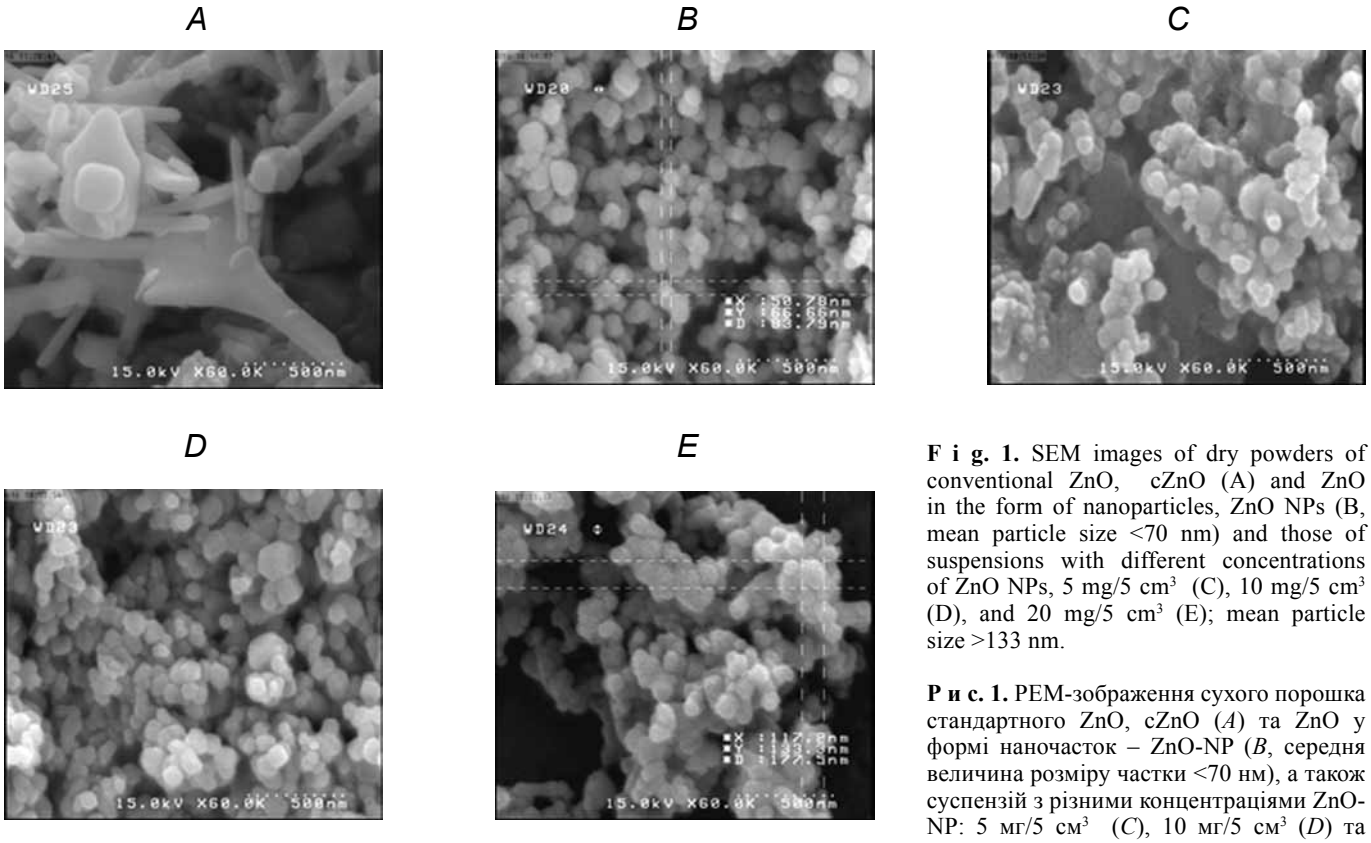
Animals and Treatment. Male albino Wistar rats weighing, on average, 200 ± 20 g were obtained from the animal house of the Joundi Shapoor Medical Sciences University and accommodated for more than a week in a room at 24 ± 1°C with a controlled 12/12 h light–dark cycle (experiments were carried out during the light phase of the cycle). The animals were housed in polypropylene cages (four per cage). Food and drinking water were freely available (except during brief test periods). In each experiment, eight animals were used; each animal was used once only. The drugs used in the study were ZnO NPs (<70 nm, Lolitec, Germany) and cZnO (Merk, Germany). Nano ZnO and cZnO suspensions were prepared by sonication for 16 min in an ultrasonic bath; before each injection, the suspensions were shaken for 1 min. Drugs were injected i.p. at doses of 5, 10, and 20 mg/kg, while the control group received 0.9% saline (1.0 ml/kg) [19].

The size and aggregation of ZnO NPs were estimated in dry powder and suspension by scanning electron microscopy (Hitachi S4160, Japan), and pH values were measured by a pH meter calibrated in buffers.

Elevated Plus Maze (EPM). Behavioral testing was carried out in a dimly lighted room. Animals were adapted to the testing room for 1 h prior to testing. A wooden plus maze consisted of two open arms (50×10 cm) and two closed arms of the same size (with 40-cm-high end and side walls). The arms were connected by a central 10×10 cm area; there were no walls on the open arms. The height of the EPM above the floor was 50 cm. Rats were placed in the center of the EPM with their head facing an open arm, left undisturbed for 5 min, and then removed and returned to their home cages. The experimental sessions were recorded by a camera and analyzed off-line (by maze router software, Iran). A rat was considered to be on the central platform when at least two paws were on the latter and in the arm whenever all four paws were on it. The normalized values of time spent in open arms (time in open arms/time in open + closed arms) · 100% and the percentage of open arm entries, OAE (number of open arm entries/number of open + + closed arm entries) · 100% were used as measures of the anxiety level [20].The distance traveled in close and open arms during 5 min was considered a measure of locomotor activity, LA; maze router software was used. In all experiments, the interval between injections and tests was 30 min.

Zinc Content Analysis and pH Measurement in Blood Serum. By analyzing the Zn content and measuring pH in the serum 30 min and 24 h after injection, we tried to estimate effective and non-effective doses of ZnO NPs and compare them with the effective doses of cZnO. The animals were anesthetized by ether, and blood was collected by cardiac puncture. Then the serum was obtained by centrifugation of whole blood at 3,000 rpm for 20 min; pH was measured by a pH meter calibrated in a buffer, and the serum Zn content was analyzed by an atomic absorption spectrophotometer (Avanta, GBC, Australia). The spectrophotometer was calibrated every time by running at least five standard concentrations (0.25, 0.5, 0.75, 1.0, and 1.5 ppm) of zinc.

Statistical Analysis. Numerical data were expressed as means ± s.e.m. The Student's *t*-test was used



F i g. 1. SEM images of dry powders of conventional ZnO, cZnO (A) and ZnO in the form of nanoparticles, ZnO NPs (B, mean particle size <70 nm) and those of suspensions with different concentrations of ZnO NPs, 5 mg/5 cm^3 (C), 10 mg/5 cm^3 (D), and 20 mg/5 cm^3 (E); mean particle size >133 nm.

Р и с. 1. РЕМ-зображення сухого порошка стандартного ZnO, cZnO (*A*) та ZnO у формі наночасток – ZnO-NP (*B*, середня величина розміру частки <70 нм), а також суспензій з різними концентраціями ZnO-NP: 5 мг/5 $см^3$ (*C*), 10 мг/5 $см^3$ (*D*) та 20 мг/5 $см^3$ (*E*); середня величина розміру частки >133 нм.

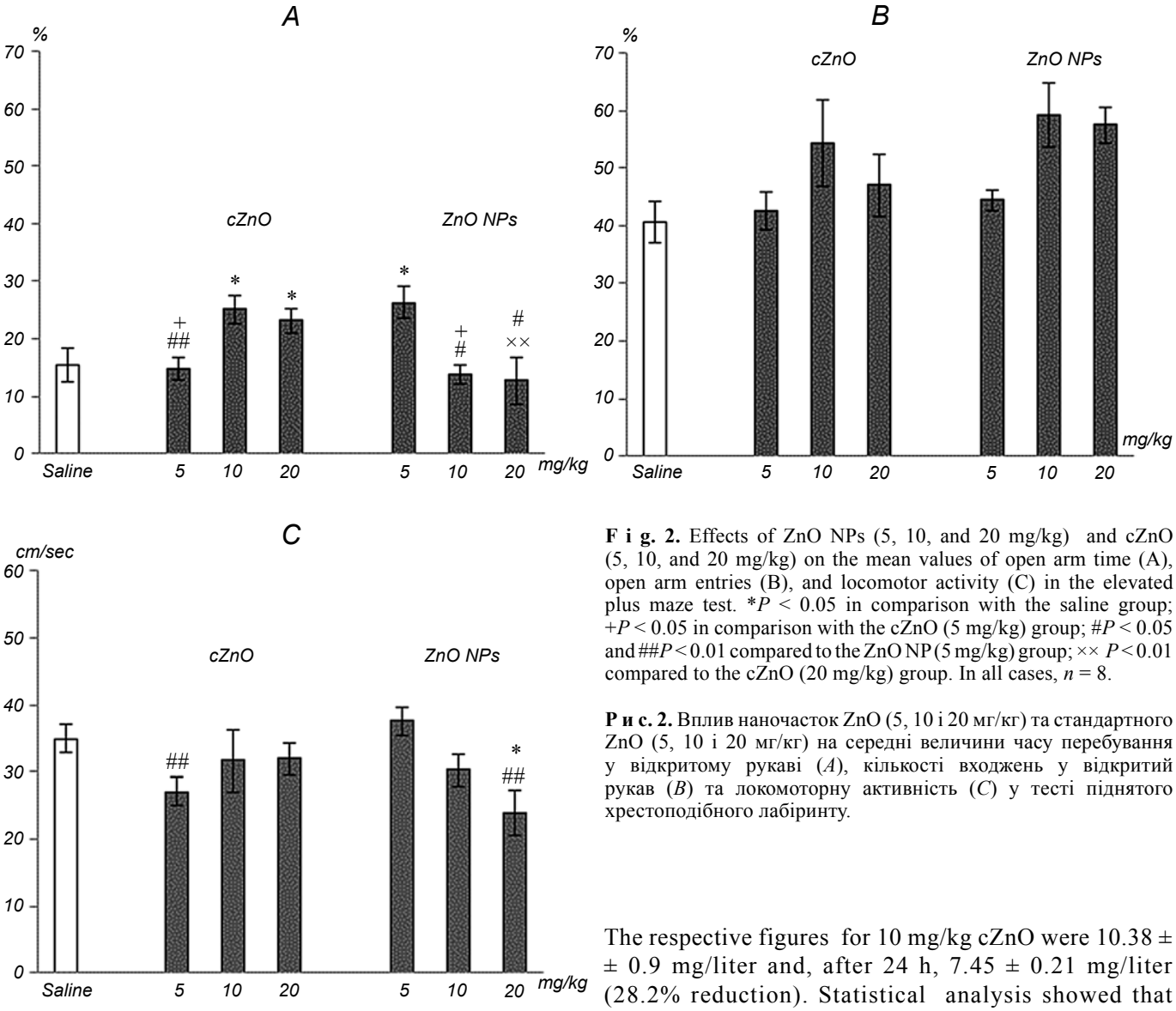
for comparison of the means of unpaired data. For multiple comparisons between groups, ANOVA was used, and the LSD *post-hoc* test was performed with InStat 3 software. Differences between experimental groups at each point with $P < 0.05$ were considered statistically significant.

RESULTS

Determination of the dimensions of ZnO NPs and pH in suspension with different concentrations according to SEM images (Fig. 1) from cZnO dry powder (A), ZnO NPs (B), and ZnO NPs at different suspension concentrations (5, 10, and 20 mg/5 cm^3) showed that the NPs tended to aggregate when the concentration was increased; the mean particle size increased from <70 nm (in dry powder) to >133 nm (at a maximum concentration, 20 mg/5 cm^3). In addition, the pH of ZnO NPs and cZnO showed acidic properties, and this acidity decreased in a dose-dependent manner.

Effect of cZnO on Anxiety-like Behavior. In Fig. 2, the effects of i.p. injections of different doses of cZnO (5, 10, and 20 mg/kg) are shown. One-way ANOVA showed significant increases in OAT ($P < 0.05$) at doses of 10 and 20 mg/kg of cZnO (increments 66 and 52%, as compared with the control). All doses of cZnO did not change considerably the LA and OAE indices (the OAE increased somewhat at 10 and 20 mg/kg but insignificantly). Thus, cZnO at a dose of 10 mg/kg exerted a maximum anxiolytic effect, and we selected this dose for the subsequent experiments.

Effect of ZnO NPs on Anxiety-Like Behavior. Figure 2 also shows the effects of i.p. injections of different doses of ZnO NPs (5, 10, and 20 mg/kg). The first of these doses provided a significant (72%) increase in the OAT ($P < 0.05$), while ZnO NPs in other doses did not increase this index. All doses of NPs did not influence significantly the OAE (a trend toward an increase was noticed at higher doses). ZnO NPs at a dose of 20 mg/kg reduced significantly LA compared with the control ($P < 0.01$). Thus, ZnO NPs at a dose of 5 mg/kg showed a clear anxiolytic



F i g. 2. Effects of ZnO NPs (5, 10, and 20 mg/kg) and cZnO (5, 10, and 20 mg/kg) on the mean values of open arm time (A), open arm entries (B), and locomotor activity (C) in the elevated plus maze test. *$P < 0.05$ in comparison with the saline group; +$P < 0.05$ in comparison with the cZnO (5 mg/kg) group; #$P < 0.05$ and ##$P < 0.01$ compared to the ZnO NP (5 mg/kg) group; ×× $P < 0.01$ compared to the cZnO (20 mg/kg) group. In all cases, $n = 8$.

Р и с. 2. Вплив наночасток ZnO (5, 10 і 20 мг/кг) та стандартного ZnO (5, 10 і 20 мг/кг) на середні величини часу перебування у відкритому рукаві (*A*), кількості входжень у відкритий рукав (*B*) та локомоторну активність (*C*) у тесті піднятого хрестоподібного лабіринту.

effect, and this dose was selected for the subsequent experiments.

Zinc Concentration and pH in the Blood Serum. There were significant differences between the serum zinc concentration and pH at all treatments in comparison with the control group; significant differences between the zinc concentration in treated groups depending on the doses or time were also observed. During the anxiety test, the mean serum zinc concentration at injections of 5 mg/kg ZnO NPs was 5.31 ± 0.53 mg/liter; after 24 h, it was reduced to 4.29 ± 0.31 mg/liter (19.21% reduction); at a dose of NPs of 10 mg/kg, this index was 22.3 ± 2.6 mg/liter. The respective figures for 10 mg/kg cZnO were 10.38 ± ± 0.9 mg/liter and, after 24 h, 7.45 ± 0.21 mg/liter (28.2% reduction). Statistical analysis showed that the regression coefficient R between the zinc serum concentration and anxiolytic effect was 44%, while between the values of pH and anxiolytic effect it was 0.4%.

DISCUSSION

The elevated plus maze (EPM) test is one of the most popular tests for all currently available animal models in neurobiological anxiety research; it is used as a screening test for putative anxiolytic or anxiogenic compounds [21]. Our results showed that acute injection of ZnO NPs in the lowest dose (5 mg/kg) and cZnO in greater doses (10 and 20 mg/kg) induced anxiolytic effects in the EPM test in adult male rats.

Some studies agree with our results. For example, it was shown that dietary zinc deficiency in mice induced anxiety-related behavior in the novelty-suppressed feeding test measured as enhanced latencies to eat [8, 9]. It was also shown that 2-month-long feeding with high levels of zinc supplements, such as zinc methionine, $ZnSO_4$, and ZnO, reduced anxiety in rats subjected to the EPM test [10].

Figure 2 shows that equal doses of ZnO NPs and cZnO exert dissimilar effects on anxiety behavior, and the efficacies of equal doses do not overlap. The anxiolytic dose of ZnO NPs corresponds to half of the anxiolytic dose of cZnO; 5 mg/kg cZnO exerts no effect on anxiety. These differences may be due to the small size of ZnO NPs and different physicochemical properties in comparison with those of the conventional form. As a result, ZnO NPs possessing greater mobility and uptake across biological membranes can interact more strongly with biological tissues [22, 23]. The greater surface area of nanoparticles provides more reactive groups and higher reactivities than the conventional ZnO form [24].

Our data also showed that all doses of cZnO and 5 and 10 mg/kg ZnO NPs did not change significantly LA, while 20 mg/kg ZnO NPs reduced this activity with practically no change in anxiety-like behavior. Thus, the anxiolytic effects of cZnO and ZnO NPs are not related to of LA modulation. Higher doses of ZnO NPs increased significantly the serum zinc concentration but provided no changes in the anxiety indices. This finding indicates that the lowest dose of ZnO NPs used released enough zinc to act on the respective receptor structures; moreover, higher doses are likely to saturate the serum zinc level but reduce the anxiolytic effect, which agrees with our previous results [25].

We found that in all treated groups the pH increased in comparison to that in the control group, but there were no significant differences between pH in all treated groups. Furthermore, statistical analysis did not show any correlation between the anxiety parameters and pH. This fact indicates that increased pH did not affect the anxiety level in our study. After 24 h, the reduction of the zinc concentration in the ZnO NP groups was lower than that of cZnO; thus, it seems that the clearance of ZnO NPs is less than that of cZnO [25]. Thus, the efficacy of ZnO NPs with respect to the reduction of anxiety may depend on the ZnO structure and longer bioavailability more than on its concentration [26]. In addition, SEM images showed that, with rising aggregation at higher ZnO NP concentrations, the anxiolytic effects decreased. Thus, ZnO NP pharmacokinetics need more investigation [25].

Our unpublished data also showed that, 10 days after acute administration of these drugs, there were no mortality, loss of weight, or considerable visible impairments in the health status of the animals, suggesting that injections of these doses of the drugs are at least not lethal.

The anxiolytic effect of ZnO NPs or cZnO may be due to the role of zinc in the anxiety-related neurochemical systems. In presynaptic spaces, zinc is co-released with glutamate, and zinc is an inhibitory neuromodulator in the glutamate signalling system [27, 28]. A number of studies indicated that glutamate is an important factor in the formation of anxiety and anxious behavior. Blocking of glutamate NMDA receptors can elicit a significant anxiolytic effect [29]. Several studies demonstrated that stimulation of NMDA receptors induces anxiogenic-like behaviors in a variety of animal models of anxiety [30]. Administration of competitive and noncompetitive NMDA receptor antagonists induced anxiolytic behaviors in humans and laboratory animals [30]. Electrophysiological studies showed that zinc weakens the NMDA receptor-mediated response via two different mechanisms, voltage-independent non-competitive (allosteric) inhibition responsible for reducing the channel opening frequency, and voltage-dependent inhibition representing the open-channel blocking effect of zinc [31]. Therefore, the release of zinc together with glutamate reduces the ability of glutamate to activate postsynaptic NMDA receptors.

Interestingly, Li et al. [32] showed that, in the experiments combining zinc imaging with electrophysiological recording during electrical stimulation of rat hippocampus *CA3* slices, the release of zinc from mossy fiber terminals occurs in a certain synaptic region of the dendrites (*stratum lucidum*) that is rich with NMDA receptors. Therefore, we suggest that exposure to ZnO particles inhibits glutamate influences on NMDA receptors (an important neurochemical system is anxious animals).

Another alternative way to decrease the glutamate output in the CNS can be achieved by intensification of GABA neurotransmission. A balance between GABA receptor-mediated inhibition and glutamate receptor-mediated excitation can regulate behavioral and physiological responses associated with anxiety [33, 34]. Zinc promotes the release of GABA from interneurons in the hippocampus, thus enhancing the inhibitory effects of this neurotransmitter and leading

to decrease in presynaptic release of glutamate [35]. As a result, the release of zinc from cZnO and ZnO NPs in our study is likely to be responsible for reducing the anxiety level via a reduction in the release of glutamate and blocking of NMDA receptor and/or via increase in the release of GABA and disrupting the balance between glutamate and an GABA in the CNS.

Thus, our study showed that the efficacy of ZnO NPs to reduce anxiety is much greater than that of conventional ZnO introduced in an equal dose. Increasing doses of ZnO NPs do not increase their efficacy from the above aspect. The beneficial effects of this ZnO form may be related to the specific size and structure or selectivity with respect to specific target.

All experimental procedures were carried out in accordance with international and institutional guidelines for animal care and use.

The authors, M. Torabi, M. Kesmati, H. E. Harooni, and H. N. Varzi, have no conflict of interests.

This study was supported by the Shahid Chamran University of Ahvaz, Iran, grants No. 90/302/18672, June 7, 2011. Hereby, researchers of this study would like to express their sincere gratitude to the Esteemed Vice-presidency for Research of Shahid Chamran University for their financial and moral supports .Thanks are due to Dr. Nahid Pour Reza and Dr. Seyyd Mansour Syednejad from the Department of Chemistry and Biology for their laboratory supports.

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ВПЛИВ ZnO У ФОРМІ НАНОЧАСТОК ТА СТАНДАРТНОГО ZnO НА ПОВ'ЯЗАНУ З ТРИВОГОЮ ПОВЕДІНКУ У ЩУРІВ

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Р е з ю м е

Як було показано, додавання мікроелементу цинку (наприклад, у формі окису цинку, ZnO) може істотно впливати на рівень тривожності. Ми досліджували вплив ZnO у формі наночасток (НЧ) порівняно зі стандартним ZnO (cZnO) на пов'язану з тривогою поведінку у щурів. Дорослі щури лінії Вістар були поділені на сім груп – контрольну групу (тварини отримували фізіологічний розчин) та шість груп щурів, котрі отримували 5, 10 та 20 мг/кг ZnO-НЧ, а також 5, 10 та 20 мг/кг cZnO. Усі диспергровані у 0.9 %-му фізіологічному розчині препарати ін'єкували внутрішньоочеревинно; через 30 хв після ін'єкції рівень тривоги оцінювали згідно з результатами тесту піднятого хрестоподібного лабіринту. ZnO-НЧ (5 мг/кг) і cZnO (10 та 20 мг/кг) істотно збільшували нормовані величини часу перебування у відкритих рукавах (%) порівняно з таким показником у контрольній групі ($P < 0.05$). Це свідчить про анксіолітичні ефекти обох агентів; крім того, ZnO-НЧ (20 мг/кг) послаблював локомоторну активність ($P < 0.05$). Концентрація цинку в сироватці при дії агентів у анксіолітичних дозах була багаторазово збільшеною. Усі дози збільшували pH сироватки до 8.05–8.10 та підтримували цей показник на постійному рівні протягом 24 год. Наші результати вказують на те, що інтенсивність анксіолітичного ефекту ZnO-НЧ є набагато більшою, ніж інтенсивність дії стандартної форми ZnO. Застосування ZnO-НЧ у якості нового терапевтичного препарату у разі тривожних неврозів потребує подальшого дослідження.

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