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RESEARCH ARTICLE

Behavioural multigenerational effects induced by the administration of very low doses of zinc during pregnancy, lactation, and prepuberal period in the rat

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ABSTRACT

In studies from this laboratory, the chronic administration of ZnTe during pregnancy, lactation, and prepuberal stages of litter (F_1 generation) modified the behavioral patterns of motivated exploration, lateralized exploration, social activity, and survival responses of maturing rats. To determine whether these affected behaviors would extend to the next generation, F_1 litter rats previously exposed to tellurium (Te) up to 30-day-old were left at rest with no further treatment up to 90-day-old. Then, F_1 female rats were mated with normal untreated male rats, and in the next generation (F_2), the litter rats at 30-day-old preserved the modified behaviors previously observed in their parents. The study revealed that Te effects were intergenerational. Here, considering that ZnTe was used in the previous study and that Zn ion has many physiological functions in the cell, experiments were conducted to elucidate if Zn would have an intergenerational effect similar to Te. Working with the same experimental setup as in the previous study but using $ZnCl_2$ instead of ZnTe, results revealed that none of the behavioral responses studied were affected by the F_1 generation. However, in the F_2 generation, lateralized exploration and survival behavior were inhibited, suggesting that Zn also has an intergenerational effect.

1 Introduction

Zinc (Zn) is a trace element that humans earlier used to galvanize iron and steel against metals' natural corrosion. This industrial application remains to date [1, 2]. It took several years for the scientific community to appreciate this element's vital role as an essential nutrient in medicine and biology [2, 3]. Zn participates in many critical oxidation-reduction reactions in

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normal cellular metabolism [2–4]. This trace metal has been found as a prosthetic group for several crucial enzymes, such as carbonic anhydrase, phospholipase C, alkaline phosphatase, and other cellular regulatory enzymes [3, 5]. Another key participation of this bivalent trace element in cellular homeostatic reactions is the formation of Zn finger proteins, a group of cell molecules that regulate gene expression [4, 6]. Alternatively, Zn is involved in the regulation of sleep [7], anxiety, and depression [8], and maintenance and regeneration of intestinal epithelial tissue [9]. This evidence emphasizes the versatile and crucial role of Zn cell physiology.

Stability and perpetuation in time are ubiquitous characteristics of living systems. Reproduction and transference of all basic information to the next generation are performed by DNA molecule codification (encoding). Thus, in nature, perfect transgenerational information is an evolutionary means to maintain species stability and perpetuation. Considering that Zn is a versatile essential bioelement with a wide spectrum of cellular actions, its participation as a Zn finger protein is related to DNA regulation. However, whether this element has a possible role in the molecular mechanisms of inheritance is questionable. In a study from our laboratory with ZnTe administered during pregnancy and lactation in nontoxic, low doses to mother rats, the F_1 generation that displayed several behavioral parameters related to natural cognitive responses was affected [10, 11]. One problem with this evidence is that ZnTe simultaneously contains Zn and Te. Since Te epigenetically affects the final behavioral expression previously mentioned, Zn's possible participation was unclear [12, 13]. This study presents the same experimental design previously described [13] but using zinc chloride ($ZnCl_2$) instead of K_2TeO_3 to define Zn's probable role in these multigenerational processes.

2 Materials and methods

2.1 Animals

Rats of a Holzman-derived colony, 30-day-old with no distinction of sex, maintained under thermoregulated (22–24 °C) and controlled light conditions (On 6:00/Off 20:00) were used. Standard rat chow and water were available *ad libitum* to the control group. In the test groups, animals had access to the trace element with no restrictions to the standard rat chow.

2.2 Experimental design

The experimental protocol used in this study has been previously described [12]. Briefly, chronic exposure to Zn, beginning from mother rat fertilization up to prepuberal maturation stages of litter rats, was conducted. From 35-day-old to 90-day-old, the trace element-treated animals (F_1) remained at rest without further treatments (Fig. 1). At 90 days of age, female F_1 generation rats were mated with normal male rats, yielding the F_2 generation.

There were three experimental groups:

(1) Control animals (no trace element treatment, water only, $n = 11$)

(2) F_1 animals (with $ZnCl_2$ treatment, $n = 10$)

(3) F_2 animals (no $ZnCl_2$ treatment in the F_2 generation, $n = 20$)

As formerly specified, pups were standardized to 10–12 animals per litter to maintain whenever possible the relationship of 1:1 of male to female rats at birth for all groups. Thus, there were initially approximately ten animals for each group in the behavioral tests. When maturing rats were 21-day-old (treatment day 42), young rats were weaned and separated from their mothers. Here,

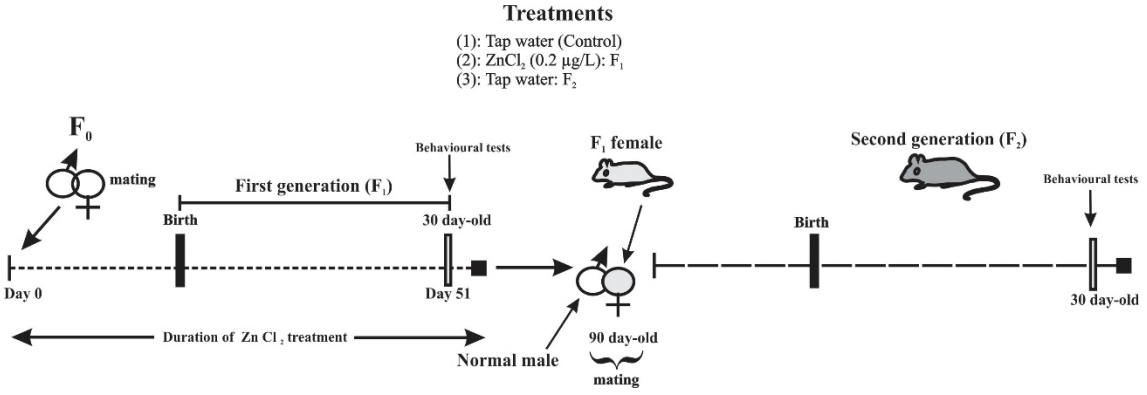

Fig. 1 Experimental design to the study of Zn effects across generations in rats. F_1 = first generation; F_2 = second generation. Animals are exposed to $ZnCl_2$ after mating, during pregnancy, lactation and up to litter rats reached 30-day-old. F_0 and F_1 are exposed to the Zn treatment. At 90-day-old, F_1-female is mated with a normal male. The pregnant rat then received no further treatment. Further details, see material and methods.

the mother rats were discarded from the experiment. At 30 days old (treatment day 51), young rats of both sexes were subjected to behavioral tests to evaluate general motor activity, motivated lateralized exploration, socialization and defensive behaviors, as previously described [10]. After that, all F_1 animals remained at rest with water and chow pellets *ad libitum,* and no further experimental treatment was applied. When F_1 rats reached 90-day-old, female rats were mated with normal rats. After that, pregnancy, delivery, lactation, and maturation of the F_2 litters were maintained with no treatment until the rats were 30-day-old when the behavioral tests were performed, as previously described [10]. At the end of the experiment, the animals were sacrificed by lethal intraperitoneal (i.p.) injections of sodium pentobarbital (40 g/100) and sodium diphenylhydantoin (5 g/100, Euthanyle, Brouwer Inc, Argentina).

2.3 Chemicals

$ZnCl_2$ (Tetrahedron, Laboratorio Andes, Industria Argentina) was used.

2.4 Behavioral tests

As previously mentioned, ZnTe administered in the chronic scheme induced modifications in several behavioral responses related to cognitive functions that lasted to the next generation [13]. To evaluate if $ZnCl_2$ could affect the same behavioral responses, the following tests to evaluate motivated exploration, lateralized exploration of novel environments, social interaction, and defensive behavior were performed.

2.4.1 The general activity and exploratory behavior detector (OVM)

It consists of a rectangular open field with acrylic walls, equipped with infrared detectors and digital counting devices to measure animal activity (Optovarimex instruments, U.S.A). The device was enriched with holes in the floor and a tube rack as a novel object, as previously described [10].

The following variables were measured to evaluate exploration motivated by novelty.

(1) Head-dipping: the number of times the animal dips its head to ear level into any OVM floor hole.

(2) Rearing: the number of times the animal rears on any lateral wall of the OVM with its anterior arms, lifting its head, or leaning on its rear legs on the floor.

(3) Focalized exploration: the duration of

exploratory behavior dedicated to the novel object located in the OVM center, measured by a digital counter at a rate of 2 counts per second. The test was applied to single animals and had a total duration of 5 min.

2.4.2 The double lateral hole-board labyrinth (DHBL)

This labyrinth evaluates motivated exploration expressed in lateralized form, as previously described [10, 14].

DHBL is made of wood and is composed of a rectangular cage 39 cm in width, 70 cm in length, and 15 cm in height. Inside, two compartments are disposed of at 90°. The first compartment (initial) is 39 cm in length and 15 cm in width, with a central entrance to the second compartment (corridor). The corridor has a length of 55 cm, 17 cm in width, and on its sidewalls, there are 4 lateral holes, each 3 cm in diameter. In this test, animals' behavioral activity was driven only by exploratory motivation induced by novel environments. The following variables were measured:

(1) Lateralized exploration. In this variable, all behaviors related to exploration are displayed when the animal chooses one side of the corridor during exploration. Behaviors include the following: (i) walking near the left or right corridor wall, at a constant speed, with vibrissae touching the wall; (ii) lateral head-dipping; (iii) rearing against the left or right corridor wall: this score was measured in the same way as the corridor behavioral activity.

(2) Percentage of animals showing left-biased exploration. Calculated by counting the number of rats showing left preference following the total number of animals tested.

Non-exploratory activities, such as immobilization at any corridor site, walking at the center, not approaching any side wall, or grooming, were not measured.

In this test, behavioral laterality was considered present when the median lateralized exploration on one side of the walls statistically outnumbers the opposite exploration. The behavioral activity was measured using an automatic digital counter (counting rate 2 counts/s) monitored by an observer unaware of the treatments. The test was applied to single animals and lasted for 3 min.

2.4.3 The social interaction test

This test (intruder–host territorial test) measures two interacting rats' social display in a determined territory challenge by an intruder [11]. The test was performed in a rectangular steel cage (26-cm width, 42-cm length, and 20-cm height) with wood shavings on the floor. This test lasted for 5 min. In the two initial minutes, the test animal (host rat) was left alone in the arena to familiarize themselves with the cage. At 3 min, a new rat of the same size and sex (intruder rat) was introduced in one corner of the cage. The behavioral display was recorded until the testing period ended. The following variables were measured:

(1) Latency to interact: the time taken (using a digital counter) for the host animal to face the intruder (α-behavior). Sniffing, touching, gentle biting, and dragging the intruder were recorded as social, behavioral displays.

(2) Duration of α-contact: the time taken (using a digital counter) for α-social interaction as displayed by the host animal in the test.

2.4.4 Forced swimming test

This measures animals' defensive behavioral response to a stressful situation represented by active swimming in a closed environment with no escape [10]. The device consists of a transparent acrylic tube measuring 50 cm height by 12 cm diameter (internal diameter), filled with water at room temperature up to half the cylinder height. Two variables were measured.

(1) Active swimming activity: the vigorous swimming movements displayed by animals involving all four extremities at an approximately constant rate, and motor activity displayed during immersion indicative of escape. The activity was measured using an automatic digital counter at a rate of 2 counts/s monitored by an expert observer unaware of the treatment.

(2) Immobilization: the time-lapse whereby animals do not swim. They float without movement or display a slow motion of their extremities enough to avoid drowning. Since the test lasted for 3 min (360 counts), this behavioral activity was obtained by subtracting the total count's active swimming activity.

All behavioral tests were filmed using a digital video camera and recorded in a DVD player/recorder (Phillips, model DVDR3455H) at an artificial illumination of approximately 180–206 lux.

Results of the behavioral measures are expressed as counts/3 min (C/3 min).

2.5 Statistical analyses

Multiple comparisons for behaviors between experimental situations were performed by non-parametric Dunn's test [15]. When comparisons involved paired groups, the Mann–Whitney *U*-test was used. The significance of single percentage differences was analyzed by a binomial distribution (the sign test). A p-value < 0.05 was considered statistically significant. Results are presented as the median ± standard error of the median

2.6 Animals' ethical care

This experimental protocol followed the recommendations of the *Guide for the Care and Use of Laboratory Animals (8th edition)*, NIH [16], and guidelines by C. J. Foltz [17].

Whenever possible, the number of animals was reduced to the acceptable minimum, thereby allowing statistical discrimination in the experiment.

3 Results

3.1 Motivated exploration in the OVM

The three primary behaviors related to exploratory motivation induced by novelty in F_1 and F_2 maturing rats are shown in Fig. 2. Treatment with Zn unaffected head-dipping, rearing, or focalized exploration in F_1 and F_2 rats (Fig. 2, Panel 1) compared with the control.

3.2 Lateralized exploration in the DHBL

In the control F_1, and F_2 generations, the lateralized exploration was measured in the DHBL (Fig. 3). Treatment with Zn unaffected normal spontaneous left-biased exploration in the F_1 animals as seen in control rats [Fig. 3(A)]. However, in the F_2 generation, left-biased exploration was absent [Fig. 3(A)]. When the percentage of animals showing left-biased exploration was examined in the three groups, only F_2 rats showed random exploratory choice (no preference for any sidewall), while 80% of

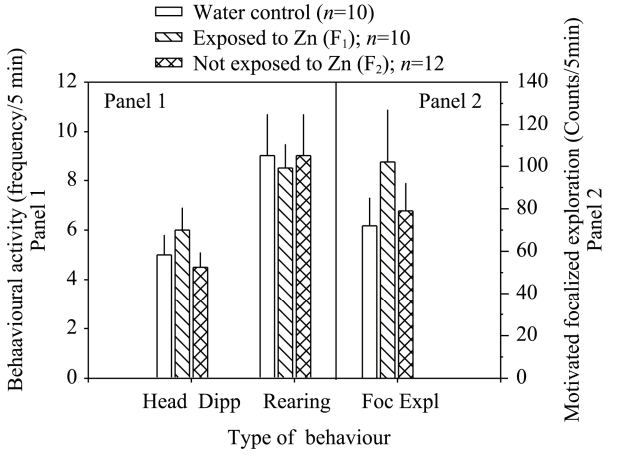

Fig. 2 Behavioural responses of rats exposed to $ZnCl_2$ (F_1), not exposed to $ZnCl_2$ (F_2), and control (water) animals in the enriched open field (OVM). Head Dipp = head dipping; Foc Expl = focalized exploration.

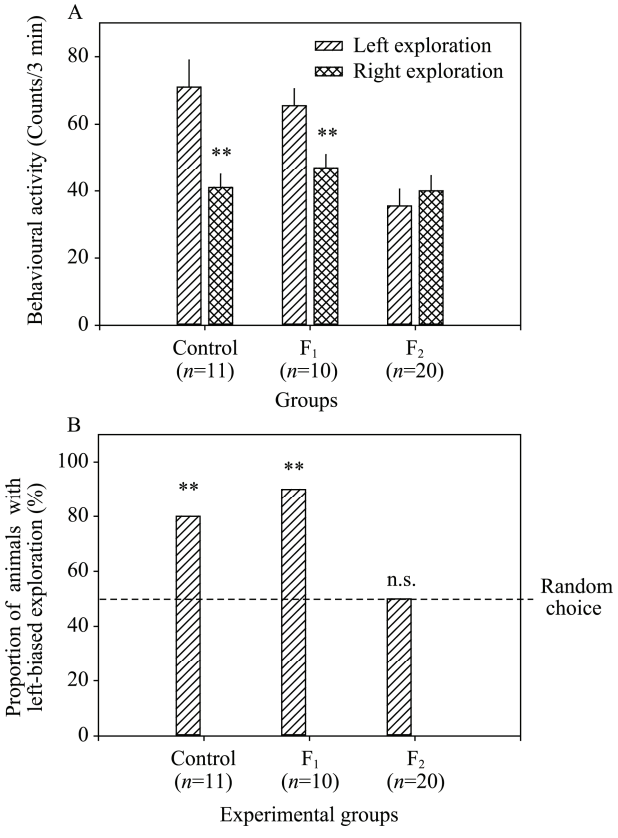

Fig. 3 Lateralized exploration in the DHBL of rats exposed to $ZnCl_2$ in the first generation. (A) Left and right exploration for the three experimental groups. ** $p < 0.01$ compared to left-biased exploration. (B) Proportion of rats with left-biased exploration in the DHBL. ** $p < 0.01$ compared to 50% which indicate no lateralization or random responses.

the control and 90% of the F_1 animals showed left exploratory preferences [Fig. 3(B)]

3.3 Social activity in the intruder—resident test

The social interaction activity is displayed when an intruder rat is introduced into a resident animal's cage for the three experimental groups (Fig. 4).

Control animals took approximately 30.5 ± 7 counts/3 min of latency to confront the intruder rat. No significant differences compared to the control was found in the F1 and F2 groups (Fig. 4).

3.4 Survival behavior in the forced swimming test

The active swimming in the escape response and

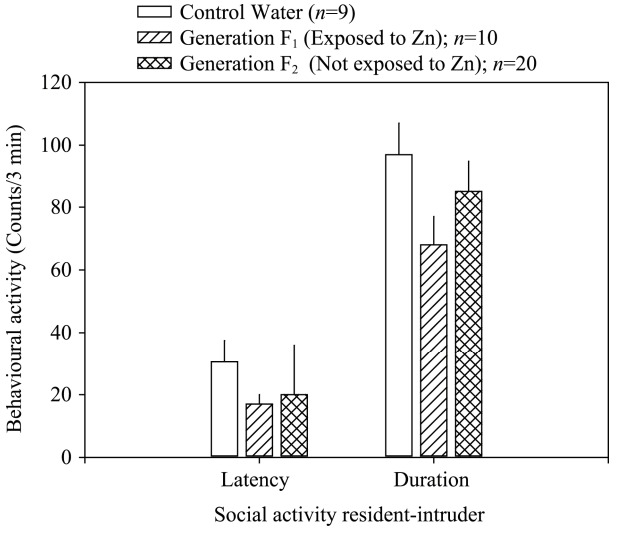

Fig. 4 Social activity parameters in rats exposed to $ZnCl_2$ in the first generation. Additional details, in materials and method section.

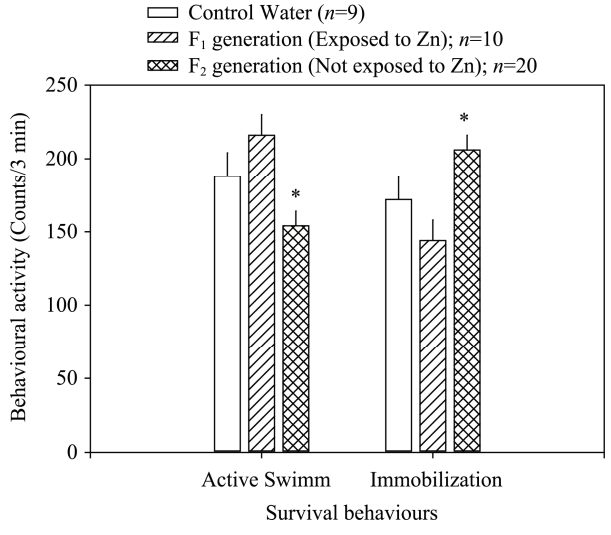

Fig. 5 Survival behavioural responses in the forced swimming test of rats exposed to $ZnCl_2$ in the first generation. * $p < 0.05$ compared to control rats.

immobility behavior for all three groups is shown in Fig. 5.

Active swimming in the F_1 Zn-treated group was unaffected by the trace element treatment since the behavior was not statistically different from the control group (Fig. 5). However, in the F_2 group (no Zn treatment), the active swimming score decreased significantly compared with the control group. Regarding the immobilization behavior, the only group that showed a

significantly increased score than the control group was the F_2 generation (no Zn treatment, Fig. 5).

4 Discussion

In the OVM general test of motivated exploration, none of the exploratory behaviors were modified by Zn treatment in F_1 and F_2 generations (Fig. 2). Thus, this element unaffected the rat's guided motivated exploration, suggesting an absence of a multigenerational effect. This evidence agrees with previous results using ZnTe or $ZnCl_2$ administered only in the F_1 generation [10, 11].

Lateralizing exploration, such as measured in the DHBL, presented a different picture when the behavior was examined through successive generations. F_1 generation was insensitive to Zn treatment since left-biased exploration in animals treated with the trace element was similar to control rats [Fig. 3(A)]. However, in the next generation (F_2), lateralized exploratory behavior [Fig. 3(A)] and the normal percentage of rats with left-biased exploration [Fig. 3(B)] were abolished.

This effect cannot have attributed to some residual Zn concentration in their parents' blood since F_1 parent rats remained at rest without further treatment for 60 days. Considering that no mechanism to store the bio-metal in the cell is known [3], the 60 days of resting were sufficient to remove any trace of the body's bio-metal.

On the same line, behavioral survival response, a critical response to acute stress in the swimming test, was also inhibited in the F_2 generation despite being normal in the F_1 (Fig. 5). This evidence reveals that a chemical agent that does not modify several behavioral parameters in one generation does not mean that its action is harmless. Thus, it can be assumed that Zn's behavioral effects can be considered a transgenerational consequence of single metal treatment in F_1. The possibility that this inhibitory action on lateralized exploration found in the F_2 rats might be due to other random unspecific actions unrelated to an inheritance mechanism seems unsupported by the experimental data. A motivated exploration in the OVM and social interaction of rats under the same experimental conditions in the F_2 generation were unaffected by Zn treatment on F_1 (Figs. 2 and 4), suggesting altered behavioral responses (Figs. 3 and 5) were specific consequences of Zn treatment.

Comparing these results with the intergenerational effects of tellurium (Te) previously observed using the same variables [12], striking differences are evident. Te treatment affected head-dipping, rearing, lateralizing exploration, social activity, and survival behaviors in F_1 and F_2 generations [12]. Te and Zn act by different brain mechanisms to influence the behavioral responses observed in F_1 and F_2 generations. The specific brain processes whereby Zn produces the behavioral changes observed in this study are unknown. Zn has complex participation in many molecular processes essential to cell functioning [3, 7, 9, 18–20]. Another interesting point from these results is that whatever the intrinsic mechanism the trace element may have, it is fully expressed in the next generation through the F_1 mother rat since the male partner at mating is a normal male unexposed to the Zn treatment. This eliminates the possibility of the male germinal line contribution. Results also suggest the possibility of fetal reprogramming occurring after Zn exposure in the mother F_0 and F_1 generations.

Zn is involved in finger domain molecules, thereby directly interacting with DNA [20] or histone nuclear proteins [21, 22], molecular targets that modulate DNA expression.

However, since the mechanisms in influencing the behavioral expression remain unclear, only future research will likely give a more precise description of Zn cellular activities.

Conflict of interests

The authors declare that they have no competing interests in this work.

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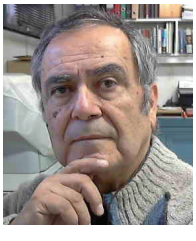

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