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Biological Effects of Trace Elements on Lateralized Exploratory Activity, Defensive Behaviour, and Epigenetic DNA Molecular Changes in Maturing Rats

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In a previous study, children of a determined geographical zone, characterized by the abundance of mineral deposits in La Rioja, province of Argentina, were found to have altered phenotypic expression attributed to the *HSR* gene. This gene has been found to be associated to handedness, brain asymmetry, reading-writing abilities and susceptibility to schizophrenia. A hypothesis was raised considering the epigenetic regulation of the *HSR* gene and its susceptibility to environmental influences; trace elements abundant in this region could be exogenous factors involved in the altered expression attributed to this gene. Thus, the objective of the present work was to test in a cognitive lateralization and associate behavioural responses rat model, the possible biological effect of ZnTe as representative trace element on some spontaneous and natural behavioural responses simile to *HSR* expression of humans. ZnTe treatment (0.03 μg/L–3 μg/L) was applied in drinking water to pregnant mother animals along all gestation, delivery, weaning and preadolescent periods. Results showed that ZnTe treatment produced two opposing effects. On one hand, treated young rats displayed excitatory motor and selective motivated exploration responses in a behavioural automatic activity device, and on the other hand, impairments in motivated and lateralized behavioural display in a lateralized exploratory labyrinth. In other different test measuring defensive behaviour, natural defensive responses were attenuated in ZnTe treated rats. Biochemical determinations of the methylation patterns of DNA in prefrontal cortex and hippocampus, showed that ZnTe treatment modified the ratio of non-methylated- to methylated cytosine, suggesting an epigenetic change on the same line to that observed in children in the previous study of this laboratory. In conclusion, the behavioural rat model used in the present study confirms some of the previous evidence found in humans.

Keywords: Defensive Behaviour, Lateralization, Trace Elements, ZnTe, Cytosine, Methyl Cytosine, Epigenetic Changes.

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1. INTRODUCTION

Trace elements are a group of chemical elements present in very low concentrations in soil and water in the world.[28,32,35] In spite that some of the metalloids composing trace elements (IUPAC Periodic Table Group 15, As, Sb, Bi, and Group 16, Se, Te) have been known for industry and agriculture since many years ago, only recently the scientific community has been aware that trace elements appear to play an important biological function in living systems.[7] Selenium (Se), for instance that initially was considered a toxic element for human health is present in a special type of proteins, the selenoproteins, where it is specifically incorporated into the amino-acid cysteine, forming selenocysteine.[9] It is interesting to note that several selenoproteins are expressed in the brain, suggesting that this type of proteins might be participating in selected brain functions, not specifically elucidated at the present time.[9,25] Se also has been found to be critical to immunological and thyroid functions in vertebrates.[21,22]

Other examples are illustrated by zinc, which it has been found to be needed for brain development, and its deficiency induces impairment of neuronal precursor cell proliferation in the rat.[10] Zinc, also stabilizes the

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finger-loop domain in DNA-binding proteins, suggesting a sophisticated molecular control on the genetic code carrier molecule of DNA.[5]

On the other hand, tellurium (Te), which in the diet is readily absorbed into the body in form of organic compounds, such as telluro-methionine giving trace concentrations of Te in body fluids,[20, 24, 33] a surprising lack of equivalent knowledge exists about its possible biological role compared to the information available about Se and others metalloids. Although Te has a lower crustal abundance, lower oxyanion solubility and biospheric mobility, characteristics that predict a low interaction with living systems, it is present in some plants of the *Alium* family such as garlic.[8] Thus, in addition to constitute a potential natural input of the metalloid in the diet, Te is responsible of the garlic-like odor in the breath of human and dogs exposed to TeO_3^{-2}.[8] So far, there is no evidence that Te is an essential element to the human body.[8] Nevertheless, toxic concentrations of this metalloid are able to selectively disrupt transcription of myelin proteins at the gene level during myelinogenesis in the brain.[13, 34] In spite that many biological inorganic chemists think that eventually scientific research will find that Te is an essential element,[8] its possible physiological role in living systems is still an unresolved matter.

One striking constant characteristic that trace elements have in general is its ubiquitous presence in all type of environments. Thus, it is not unreasonable to consider that there is a continuous interaction between these elements and living beings because of the necessary exposition due to feeding habits and water ingestion. Also it is deduced that if local geographic variation in trace elements concentration is produced, it is quite possible that changes in physiological parameters can be observed in those living beings inhabiting the region. In a previous work, these assumptions appeared to be met because our laboratory showed evidence that primary school children living in a geographical region characterized by mountains and mineral deposits in La Rioja province, Argentina (identified as "Region 2"), showed altered phenotypical manifestations attributed to the *HSR* gene compared to children of a control zone ("Region 1") in the same province.[30] It is thought that *HSR* controls the expression of handedness, brain lateral asymmetry, cognitive ability for writing-reading, and susceptibility to develop schizophrenia in humans.[16, 17, 30] Furthermore, examining the DNA methylation patterns from blood of children from these two regions, it was found that ratio of non methylated to methylated cytosine in DNA was statistically higher in those children coming from Region 2 compared to control children of Region 1.[29] This finding was of particular importance because is a significant evidence showing in a living human population that the *HSR* gene is epigenetically modulated and susceptible to environmental regulation.[18]

In this particular geographical region of La Rioja (Region 2), some of the trace elements showed atypical high concentrations in soil and water, compared to average earth crust, surface or underground waters concentrations values.[14, 19, 26, 27] For instance, Te is about 4 times more concentrated in waters of Region 2 of La Rioja than in waters from another part of the world, considered as reference.[14, 19]

According to these findings, considering La Rioja's results a hypothesis was raised that because of the unusual high concentrations of some of trace elements in water and soil of Region 2, a functional interaction of these elements with DNA is produced altering somehow the normal expression of some genes, such as the *HSR*. If this assumption is true and this epigenetic process is real, then it would be possible to reproduce in experimental animals exposed to comparable levels of trace elements. Thus, the purpose of the present work was to evaluate in rats the possible biological effects of chronic administration of zinc telluride, selected as a representative trace element, in two experimental complementary approaches: molecular determination of methylated DNA patterns and selected rat lateralized behavioural expressions.

2. MATERIAL AND METHODS

2.1. Animals

Rats of a Holzman-derived colony, weighing 250–300 g, 90 days old and maintained in thermoregulated (22–24 °C) and controlled light conditions (06.00 on-20.00 h off) were used. Standard rat chow and water were available ad libitum for control animals. Rat show ad libitum, and varying trace elements water solutions were available for treatment animals.

2.2. Experimental Design

Experimental protocol was designed in an attempt to reproduce environmental conditions found in the study of La Rioja children.[30] Chronic exposition to trace elements beginning from birth up to prepuberal maturation stages was selected as first approach (Fig. 1). Zinc telluride (ZnTe, Sigma-Aldrich Co, U.S.A.) was used as representative trace elements since these elements were found to be in high concentrations in the geographical region where *HSR* gene modified phenotypic expression.[14, 27] As shown in Figure 1 after mating, pregnant mothers were exposed to normal tap water (Control, $n = 2$); water solutions of 0.03 μg/L ZnTe (Group 2, $n = 2$); 0.3 μg/L ZnTe (Group 3, $n = 2$), and 3 μg/L (Group 4, $n = 2$). Treatments were applied during all pregnancy, delivery, lactation, weaning and prepuberal periods of maturing rats. Thus, mothers and pups were continuously exposed to ZnTe or water. At birth, pups were standardized to 10 animals per litter trying to maintain whenever when possible the relationship of 1:1 of male to female rats. When maturing rats were 21 day-old (Day 42 of treatment), young rats

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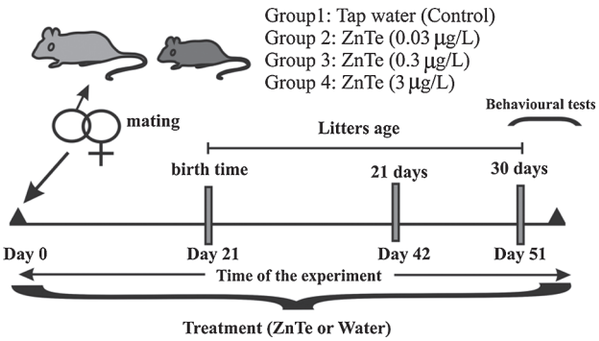

Fig. 1. Experimental design of the experiments. Four experimental groups were formed. One group was control and the others received increasing concentrations of ZnTe, administered in the drinking water. Exposition was chronic during 55 days (51 days starting from day of pregnancy up to the beginning of behavioural tests, 4 days of testing and sacrifice of animals).

were weaned and separated from their mothers. At 30 day-old (Day 51 of treatment) young rats of both sexes were subjected to a battery of behavioural tests in order to evaluate lateralized and cognitive behavioural aspects similar to some of the *HSR* phenotypic expression of humans. Total number of young rats used in all experiments was 15–20, since some animals were lost for reasons not related to experimental treatments.

After ending of behavioural tests, all animals were sacrificed by decapitation. Dissection of the brain was performed and isolation of frontal cortex and hippocampus structure was made in a total time of 30 sec at 4 °C for chemical determination of DNA.

2.3. Behavioural Tests

The following behavioural tests were used to evaluate exploration of novel environments, lateralization, preferential decisions, and defensive behaviour.

2.3.1. The General Activity and Exploratory Behaviour Detector (OVM)

It consists of rectangular open-field with acrylic walls, equipped with infra-red detectors and digital counting devices for measuring animal activity (Optovarimex instruments, U.S.A). Device was enriched with holes in the floor, and a tube rack as novelty object as described in detail previously.[4, 31]

Five variables were selected. Two of them are general estimates of motor and ambulatory activity displaying the general state of animals, and the other three are indices of motivated exploratory activity. Variables were:

(1) Ambulatory behavioural activity, the motor activity displayed by animals while they move in any direction of the arena during exploration, as measured by automatic digital counting proportional to the time of active movement.

(2) Non ambulatory behavioural activity, all movements that animals display remaining in one position, without displacement as measured by automatic digital counting proportional to the time of behavioural movement. Grooming and sniffing are the main behavioural components of this variable.

(3) Head-dipping, counted as frequency of head dips into any of the four holes of the OVM hole-board when this animal behaviour lasted at least 2 seconds.

(4) Rearing, counted as frequency of animal's rears, standing still on his rear feet and leaned on the walls of the OVM hole-board cage, sniffing to the air for at least 2 seconds.

(5) Focalized exploration, measured by digital counting proportional to time at a rate of 2 Counts/sec when the animal sniffs, touches with its front feet, climbs over the tube rack or explores the holes of the rack.

Variables (3), (4) and (5) were measured by an expert observer unaware of treatments. Test was applied to single animals and had a total duration of 5 min.

2.3.2. The Double Lateral Hole-Board Labyrinth (DHBL)

This labyrinth evaluates motivated exploration that can be expressed in lateralized form, as described previously.[1]

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

(1) Corridor behavioural activity. All behaviours displayed by rats while they are in the corridor of the labyrinth, such as walking, rearing, head-dipping, and sniffing on the left or right side walls, including non-exploratory behaviours such as grooming and immobilization measured by a digital automatic counter (counting rate 2 counts/sec) monitored by an observer unaware of treatments.

(2) Initial Compartment behavioural activity. It is included in this measure all the behavioural activity displayed by rats while they were in this compartment. This activity was not measured directly and was calculated by subtracting corridor behavioural activity counting from the total counting of the test (3 min = 360 counts).

(3) Lateralized exploration. It is included in this variable all behaviours related to exploration displayed when the animal chooses one side of the corridor during exploration. Behaviours included:

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(i) Walking nearby the left or right wall of the corridor, at constant speed, with vibrissae touching the wall.

(ii) Lateral head-dipping.

(iii) Rearing against the left or right walls of the corridor. This score was measured in the same way than Corridor Behavioural Activity.

(4) Non-exploratory activity. It is included in this variable the following behaviours:

(i) Immobilization at any site of the corridor; walking at the center of the corridor not approaching to any side wall.

(ii) Grooming. Its value was calculated by subtracting the lateralized exploratory activity from the corridor behavioral score.

In this test, behavioural laterality was considered to be present when the median of lateralized exploration on one side of the walls statistically outnumbers the opposite exploration.

Test was applied to single animals and had a total duration of 3 min.

2.3.3. Forced Swimming Test

This test measures the defensive behavioural response of animals subjected to a stressful situation represented by active swimming in a closed environment having no escape. 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 of the cylinder height. Two variables were measured.

(1) Active swimming activity, all the vigorous swimming movements displayed by animals involving all four extremities at approximately constant rate, and motor activity showed during immersion looking for a escape. Activity was measured by digital automatic counting at a rate of 2 Counts/sec monitored by an expert observer unaware of treatments.

(2) Immobilization, the time lapse where animals do not swim, floating without movements or displaying slow motion of its extremities enough to avoid sinking into the water. Since test had a total duration of 3 min (360 Counts), this behavioural activity was obtained by subtracting the active swimming activity from total counting.

All behavioural tests were filmed by with a digital video camera, and recorded in a DVD player/recorder Phillips, model DVDR3455H.

2.4. Biochemical Determinations of Cytosine/Methyl-Cytosine in Total DNA

2.4.1. DNA Extraction Procedures

DNA from frontal cortex and hippocampus of rats were extracted using a standard tissue DNA extraction kit (Tri Reagent, Sigma Chemical Company, USA). In all experiments pooled brain samples from animals were used (3–4) in order to reach over the detection limit of nucleotides in the measurement apparatus.

2'-Deoxyadenosine, 2'-deoxythymidine, 2'-deoxyguanosine, 2'-deoxycytidine and 5-methyl-2'-deoxycytidine standards were purchased from Sigma-Aldrich Química S.A. All the nucleosides were dissolved at 5 mM in Milli-Q grade water.

2.4.2. Genomic DNA Hydrolysis

DNA samples (3 μl, 0.5 μg/μl) were heated for 2 min in a boiling water bath and cooled rapidly in ice; 0.75 μl of 10 mM $ZnSO_4$ and 1.25 μl of nuclease P1 (Sigma-Aldrich Química S.A.); 200 units/ml in 30 mM $NaC_2H_3O_2$ were added and mixture were incubated for 16 h at 37 °C.

2.4.3. Cytosine and 5-Methyl Cytosine Separation and Quantification by Capillary Electrophoresis

The analysis of the hydrolyzed DNA samples was carried out as previously described.[15] The capillary electrophoresis (CE) instrument P/ACE MDQ (Beckman Coulter, Brea, CA. USA), equipped with a UV detector. Data were processed by 32 Karat™ software (Beckman Coulter). Deoxynucleoside standard solutions were daily prepared from stock solutions by appropriate dilution with water. Before their separation by CE, hydrolyzed DNA samples were carefully evaporated by means of a nitrogen stream and re-suspended in 7 μL of water. Separations were performed in an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) (60 cm in length, 75 μm i.d., 365 μm o.d.). The CE system temperature was held at 20 °C, while samples were maintained al 4 °C. Hydrodynamic injection for 10 s, 0.5 psi, was used for introduction of samples. The separation voltage was 10 kV. UV-detection at 214 nm (deuterium lamp) was performed. The running buffer and washing solutions were filtered through nylon membrane filters (0.45 μm, Micron Separations Inc., Westboro, MA, USA). The running buffer consisted of 48 mM $NaHCO_3$, pH 9.6, and 60 mM Sodium docecyl sulphate. Before each run, the capillary was washed with 1 M NaOH for 2 min, followed by 1 mM NaOH for 3 min, and running buffer for 3 min.

2.5. Statistical Analysis

To evaluate the significance of the differences in the ambulatory and non ambulatory activities between control and ZnTe groups in the OVM test, 1 way ANOVA and the Duncan test were used. To evaluate the significance of the differences in head-dipping, rearing and focalized exploration in the OVM test; corridor, initial compartment, and non exploratory activities in the DHBL; swimming and immobilization behaviours between control and ZnTe

groups, the non-parametric multiple comparisons test of Dunn was used. To evaluate left versus right exploratory activities in each treatment groups in the DHBL, the Wilcoxon test was used. The statistical significance of the difference of proportions was evaluated using the χ^2 Distribution. The significance of the differences of the ratio of non-methylated/methylated cytosine between control and ZnTe treated rat groups in the molecular analysis of DNA was evaluated using the non-parametric Mann-Whitney Test for independent observations. A probability of less than 0.05 was considered significant. All calculations were performed using a domestic standard statistical pack (PdeB, Mendoza, Argentina, Version 2.5).

Results are presented as the mean ± standard error of the mean in the ambulatory and non ambulatory behavioural activities (Fig. 2(A)), and except Figure 3(C), as the median ± standard error of the median in all the rest of measurements (Figs. 2(B), 3(A) and (B), 4 and 5).

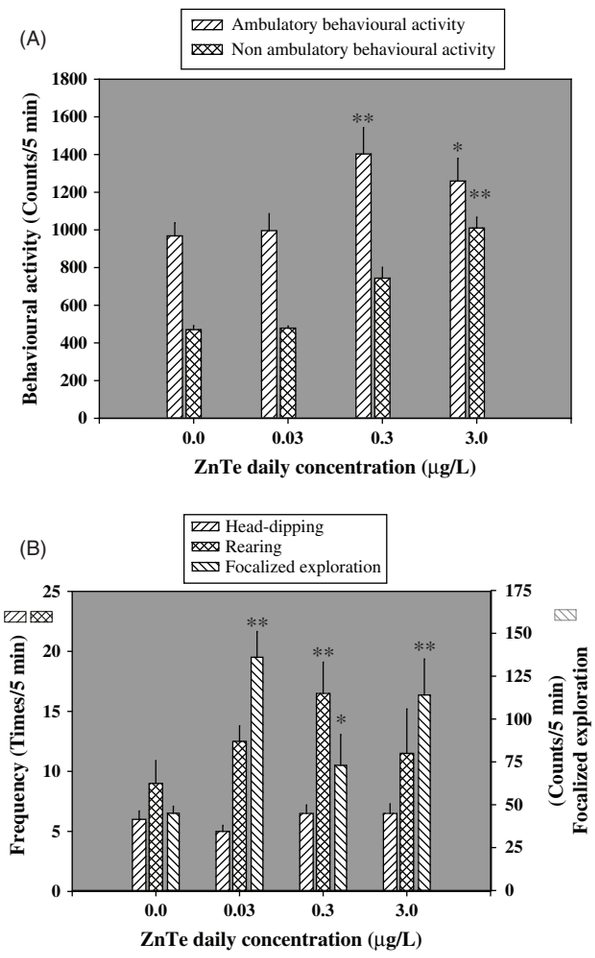

Fig. 2. General exploratory and motivated behavioural responses of rats subjected to ZnTe administration in the OVM. (A) and (B): $^{*}p < 0.05$; versus Control group; $^{**}p < 0.01$ versus Control group (dose 0). Number of animals: Control ($n = 20$); 0.03 μg/L group ($n = 15$); 0.3 μg/L group ($n = 12$); 3 μg/L group ($n = 15$) Additional details see text in Material and Methods section.

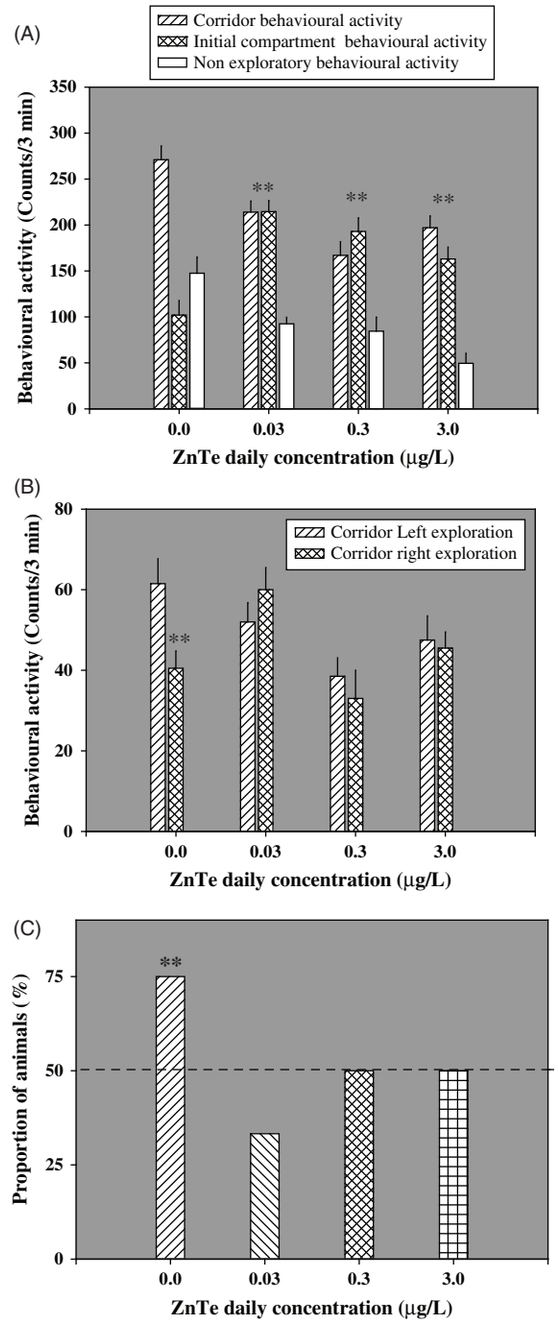

Fig. 3. Motivated and lateralized behavioural responses of rats subjected to ZnTe administration in the DHBL. (A) and (B): $^{**}p < 0.01$ versus Control group (dose 0). (C): $^{**}p < 0.01$ versus 50%. Additional details see text in Material and Methods section.

2.6. Ethical Care of Animals

The present experimental protocol was revised and approved by the Comité Institucional de Cuidado de Animales de Laboratorio (Institutional Committee of Care and Welfare of Experimental Animals) of the Faculty of Medical Sciences, Universidad Nacional de Cuyo (CICUAL).

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3. RESULTS

3.1. Daily Water Consumption During Pregnancy, Birth, Postpartum and Prepuberal Stage

Water and ZnTe solutions consumption by mothers and young rats during the treatment period is shown in Table I.

In spite of some variations, there were no differences in daily ZnTe solutions consumption of mothers and pups compared to control. Also there were no differences in ZnTe solutions consumption after weaning between the different experimental groups. No malformations at birth were noted, nor any differences in growing rate of youngster treated with ZnTe solutions. Maternal behaviour was normal for all groups.

3.2. OVM Results

The different behavioural parameters measured in the OVM test are shown in Figure 2. Control animals showed an ambulatory activity of 968 ± 70 Counts/5 min. Animals treated with 0.03 μg/L ZnTe, showed similar scores than control (Fig. 2(A)). However, young rats treated with 0.3 and 3 μg/L ZnTe, showed a significant increase in the ambulatory activity (Fig. 2(A)). With the exception of 3 μg/L dose, ZnTe treatment did not modify the non ambulatory activity of treated animals, which were similar to control values (Fig. 2(A)).

Head-dipping was not modified by the ZnTe treatments (Fig. 2(B)). Rearing in the ZnTe treated rats significantly increased over control values at the dose of 0.3 μg/L (Fig. 2(B)), and focalized exploration was statistically higher than control values in all ZnTe doses used (Fig. 2(B)).

3.3. DHBL Results

Behavioural parameters measured in the DHBL are shown in Figure 3.

Corridor behavioural activity was significantly affected by the ZnTe treatment in all doses (Fig. 3(A)). A decrease in this score was observed compared to control, and the effect did not change in spite of the increasing ZnTe dose applied to rats. At the same time, the initial compartment behaviour was increased by the ZnTe treatment (Fig. 3(A)). Non exploratory activity, which in control rats is about half of the total corridor activity, was also affected by the ZnTe treatment. A sustained significant decrease in the score was observed reaching lowest value at the dose of 3 μg/L of ZnTe.

Control rats showed a lateralized exploration of the corridor with a significant bias to the left (Fig. 3(B)). Left preference was lost in those animals treated with ZnTe. Proportion of control animals exploring left side more than right side of corridor in the DHBL was 75%, statistically different from 50% value that indicates no preference (Fig. 3(C)). ZnTe treatment abolished the left bias preference maintaining the choice of exploration at random levels in treated rats (Fig. 3(C)).

3.4. Forced Swimming Test Results

The behavioural parameters measured in the forced swimming test are shown in Figure 4.

Control rats dedicate about one third of total time to resting and floating in the water cylinder (Fig. 4). ZnTe treatment significantly decreased the active swimming, producing a corresponding increase of immobilization. Thus, the proportion of swimming to immobility was altered (Fig. 4).

3.5. DNA Methylation Patterns Results

The molecular analysis of methylation patterns of total DNA in selected brain tissues of control and ZnTe treated animals is shown in Figure 5.

Ratio of non-methylated to methylated cytosine (R_{NMM}) was about the same in the frontal cortex and hippocampus samples of control rats (Fig. 5). In the ZnTe treated animals, no change in the R_{NMM} was found in the frontal cortex sample, compared to control. However, a significant increase in R_{NMM} ($p < 0.01$, Fig. 5) was found in the hippocampal structure.

Table I. Daily water or ZnTe solutions consumption during pregnancy, delivery, lactation and weaning periods of experimental groups.

Experimental Groups[a]	Pregnancy (ml/day)	Lactation[a] (ml/day)	Lactation[b] (ml/day)	Weaning (ml/day)	Maternal behaviour score (0–5)	Offspring status at birth[d]
Control	74.3 ± 17.2 (22 days)[b]	85.5 ± 11 (10 days)	122.9 ± 6.5 (11 days)	245.5 ± 45.4 (9 days)	Normal[c]	Normal
ZnTe 0.03 μg/L	55.9 ± 2.6 (22 days)	77.1 ± 4.6 (10 days)	121 ± 5.1 (11 days)	271 ± 32.5 (9 days)	Normal	Normal
ZnTe 0.3 μg/L	49.3 ± 16 (22 days)	70 ± 6 (10 days)	121.7 ± 6.2 (11 days)	291 ± 19.2 (9 days)	Normal	Normal
ZnTe 3 μg/L	68.6 ± 4.9 (24 days)	83.7 ± 6.5 (10 days)	142.3 ± 11.3 (11 days)	277 ± 19 (9 days)	Normal	Normal

[a]Treatments were replicated twice. Puppets at birth were standardized to 10 pups per mother. Number of female and male pups in the litter, whenever possible was standardized to equal number of female and male subjects. [b]Water or ZnTe solutions consumption was relatively constant. Changes were evident after delivery; the next 10 days, when some litters also consumed liquid; the following next 11 days, when pups increased consumption of solution, and at weaning when only maturing rats consumed water or ZnTe solution. [c]When maternal behaviour scores were at least 4, it was considered that maternal display was normal. [d]At birth, pups were examined for obvious physical defects (absence o short tails, malformations of limbs, etc.).

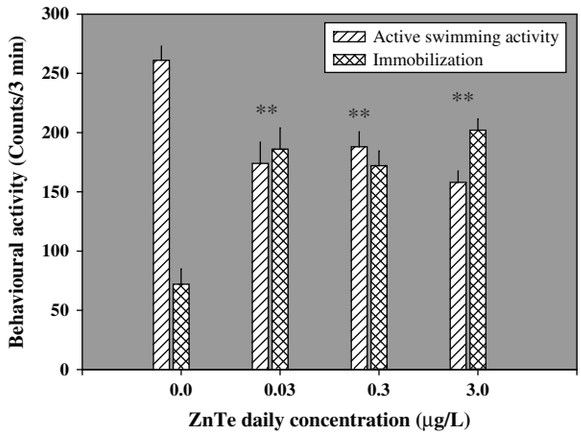

Fig. 4. Defensive behavioural responses of rats subjected to ZnTe administration in the forced swimming test. $^{**}p < 0.01$ versus Control Group. Additional details see text in Material and Methods section.

4. DISCUSSION

The basic point in this work is to have animal phenotypic expressions that can be analogized in some way to the characteristics attributed to the *HSR* expression in humans. Up to our knowledge, it has not been found an "*HSR* like" gene in rats. However, evidence exists that rat brain is lateralized and behavioural expressions in cognitive and motivational functions have bias preferences.[1,4] Since these brain properties are found to be constant from parents to offspring, a genetic background must be present. Thus, meanwhile it is not possible to affirm that these lateralized behavioural expressions can be due to an *HSR*-like gene in the rat, it is not unreasonable to speculate that a similar mechanism might be involved. Out the four phenotypic characteristics attributed to *HSR* in humans,[16,17,30] certainly writing-reading and schizophrenic susceptibility have no correlations in the animal model, because rats do not write or read, and it cannot develop schizophrenia, which is a typical mind dysfunction in man. Nevertheless, brain functional asymmetry and handedness are properties having some projecting similarities to animals.[1,4] Thus, analyzing the influence of trace elements on lateralized behavioural display in rats is an acceptable experimental model.

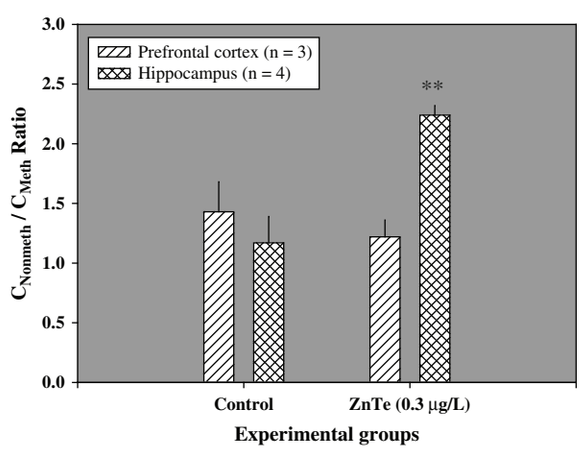

Fig. 5. Molecular methylation patterns of DNA of animals subjected to ZnTe administration after completion of all behavioural tests. $^{**}p < 0.01$ versus Control group. Additional details see text in Material and Methods section.

Te has been found to exert toxic effects during pregnancy in rats. It provokes in offspring malformations in fore-and hind limbs, absent or short tail, subcutaneous blood clots, exophthalmia and many other alterations.[34] On the other hand, Te administration impaired acquisition of a spatial learning task, provoking neuropathological changes in the hippocampus sub-fields and prefrontal cortex in adult rats.[37] These toxic Te effects are clearly related to the dose of administration. In the above mentioned studies, Te dose was 100–400 μg/Kg body weight, which in an average rat body weight of 250 g, means 25–100 μg Te/rat. In the present work, mothers with the higher Te dose received about 0.20 μg Te/rat during pregnancy (Table I), and youngsters after weaning received about 0.083 μg/rat, concentrations that were quite below the toxic levels of Te. As shown in Table I, no malformations or obvious anatomical defects were noted in the ZnTe treated rats. This evidence strongly suggests that present results can not be interpreted as the consequence of toxic Te effects.

The general physical state of animals and motivational behavioural responses to novelty were measured in the OVM apparatus (Fig. 2). Since some of the toxic effects of Te are addressed to the nervous system,[34] it was important to evaluate in the chronic Te treatment to animals, if there were some deleterious effects in the normal and natural responses of rats to novel environments. As shown in Figure 2, no adverse responses or impairments were found. These results show that Te chronic treatment had no deleterious effects on the rat nervous system in the present experimental setup. On the contrary, positive effects were noted in some behavioural responses (Fig. 2), while others were not modified. This evidence indicates that ZnTe appears to show some specificity of action in the brain because motivated behaviours such as rearing and focalized exploration were increased while head-dipping was not affected (Fig. 2(B)).

In unknown environments offering two geometrically different alternative places to explore, such as the case of the DHBL, normal rats divide its exploration according to motivational and emotional clues. Corridor of the DHBL offers two identical parallel walls with lateral holes, geometrical aspects considered attractive to the rat;[1] while the Initial compartment is completely devoid of incentive characteristics since it is made of nude walls. Control rats spend about 27% of the total activity in the Initial compartment and 73% in the corridor, suggesting that naturally motivation overcomes fear regarding corridor compartment. Treatment with ZnTe disrupts this ratio and this alteration was present even at the lowest dose used

(Fig. 3(A)). One possible explanation is to think that ZnTe interferes to those neural circuits modulating exploratory motivation, perhaps at the hippocampal level.[2,3] As complementary evidence, normal rats that have left bias exploration, ZnTe treatment abolished this lateralized tendency decreasing the proportion of rats with left exploration tendency (Figs. 3(B and C)). Lateralized exploratory responses depend on the ventral hippocampus,[1] and the possibility in the brain that one possible target may be the hippocampus is supported by this evidence.

Defensive behaviour incorporates all responses and tactics that the animal uses against an environmental challenge threatening its life, thus constituting one important mechanism of coping behaviour. Results of the forced swimming test (Fig. 4) clearly show that ZnTe treatment affected this defensive behavioural response. Brain neural circuits modulating these defensive behaviours have not been conclusively identified in the rat but it is possible that prefrontal cortex, amygdala and hippocampus may be some of the neural structures involved. Data from Figure 4 suggest that ZnTe is acting at some or all of these brain regions exerting an inhibitory modulation. It is interesting to point out that the forced swimming test in different experimental conditions has been used in rats as an index to measure depression-like signs.[6,11,12] The question if the present results are to be interpreted as a failure of brain mechanisms displaying defense behaviour, or a disruption of emotional behaviour causing depression-like signs by the ZnTe treatment is an answered matter, and certainly further research will be needed in order to disclose the role of trace elements on these processes.

Biochemical analysis of methylation patterns of DNA showed that trace element had a selective influence on brain tissue (Fig. 5). On one hand, ratio of non-methylated- to methylated cytosine in prefrontal cortex of rats treated with ZnTe was not affected. On the other hand, in these same animals, ratio R_{NMM} was almost doubled in rats treated with ZnTe. This evidence shows that trace elements in the present experimental conditions have a preference to hippocampal cells, suggesting that hippocampal structure might be a physiological target for ZnTe. Since in the present work, only two brain structures were examined, it cannot be discarded that ZnTe might be influencing others. Nevertheless, this result is in agreement with findings described by other authors using high concentrations of Te.[37] Perhaps, the most relevant conclusion is that hippocampal neuropathological effects due to high exposure to Te can not be interpreted as a simple unspecific action of the trace element,[37] because in this same structure DNA molecular changes were found at very low non-toxic concentrations of Te. Thus, Te biological effects might be more complex and subtle than expected only to a toxic effect.

The second point of relevance about the biochemical DNA effects found in this work is methylation and non-methylation of cytosine, which is a molecular sign suggesting an epigenetic change in the organism.[29] In humans, methylation changes are associated to genes having epigenetic modulation, such as the parental of origin genes, which for instance it characterizes the *HSR* locus.[16] Altered rations of non-methylated- to methylated cytosine found in the present work in rats essentially repeat the modified corresponding ratio found in children in a previous work.[29] This evidence is suggesting that something in the chromosome of rats is responding similarly to what is known about the *HSR* gene in humans under the exposure of an environmental stimulus. Thus, a third point projecting from the before mentioned arguments is that the hypothesis about children under the influence of particular geographical conditions, such as trace elements in sub-toxic but elevated concentrations[30] can explain altered molecular methylation patterns in DNA suggesting epigenetic adaptation changes modifying particular phenotypic expressions. What in our opinion is particularly relevant is that these changes are present also in animals.

As a final point, there is the quest about the biological mechanism of ZnTe actions. The present results cannot explain the intimate molecular mechanisms about how ZnTe could be acting. What is clear is that its actions are not the consequence of drastic and toxic effects on brain cells. Since Zn and Te both have biological effects on cells, there remain the question about which of these trace elements or both could be explaining the present results? Substantial biochemical and physiological evidence exists supporting a role for any of these trace elements.[9,10,21–23,36] However, in waters of Region 2 in La Rioja, Zn concentrations were not elevated compared to waters from other world zones; but Te was found to be about 4–31 times higher.[7,14,19] Even though this is not a strong argument favoring Te, it is possible to speculate that the modified behavioural expressions and molecular changes in methylation patterns can be due to biological interactions of Te with DNA. Further research, doubtless it will clear this issue.

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