RESEARCH ARTICLE

Maternal Zinc Diet Impairs Learning and Memory in Offspring Rats through the CREB/BDNF Pathway

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Abstract

BACKGROUND: Zinc released into the synaptic cleft able to modulate various signaling pathways, including brain derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB). Zinc binding to its receptor, G-protein coupled receptor 39 (GPR39), may trigger biochemical pathways leading to cAMP response element binding protein (CREB)-dependent gene transcription that subsequently promotes BDNF upregulation. Therefore, zinc dyshomeostasis should be considered as a condition that induces disruption of CREB/BDNF signaling. This study was conducted to examine the effect of maternal zinc diet on hippocampal expression levels of CREB and *BDNF* in offspring.
METHODS: One-day pregnant rats were randomly divided into five groups: zinc-deficient (D), zinc-restricted (R), zinc-adequate (A), zinc-supplemented (S), and excess zinc-supplemented (ES). The groups had different zinc diets during pregnancy and lactation. The behavioral function of the offspring was tested with Y-maze at the 43th postnatal. Hippocampus was isolated, *BDNF* was assessed by quantitative real-time polymerase chain reaction (qRT-PCR), and CREB was examined using sandwich enzyme-linked immunosorbent assay (ELISA).
RESULTS: Spatial working memory measurement demonstrated that D and ES group exhibited a significantly lower spontaneous alternation than other groups. The qRT-PCR and ELISA analysis revealed the hippocampal expression level of *BDNF* and CREB decreased in groups D and ES, but tended to increase in groups R and S, until the highest expression peak was found in group A.
CONCLUSION: High and low intake of zinc induces lower expression of *BDNF* and CREB in hippocampus, which further impairs learning and memory. Our findings suggest the signaling pathway of CREB/BDNF is involved in zinc dyshomeostasis-induced cognitive impairments.
KEYWORDS: hippocampus, BDNF, CREB, TrkB, GPR39, zinc, diet, LTP

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Introduction

Zinc deficiency is still a public health problem, especially in developing countries, and the World Health Organization (WHO) has determined zinc deficiency as the main causative factor for various diseases.(1) Worldwide, it is approximated that over 80% of pregnant women do not consume enough zinc. WHO has documented that the estimated occurrence of zinc deficiency varies from 4% to 73% across different regions, and in Southeast Asia the prevalence reaches 34-73%.(2) Zinc in a free form (Zn^{2+}) is a divalent metal ion that is abundant in the central nervous system, it is stored mainly in the vesicles of excitatory neurons, especially in the terminals of hippocampal mossy fiber neurons. The presence of zinc in hippocampus is

essential for maintaining cognitive function.(3) Zinc deficiency disorders primarily affect the developing nervous system, as the brain experiences its most rapid maturation phase during fetal development.(4) Several studies showed a correlation between maternal zinc status and children's cognitive function. Zinc deficiency may cause a decrease in zinc levels in hippocampus, which is positively correlated with spatial memory deficits in rats.(5,6) In order to prevent zinc deficiency, several efforts have been made, such as zinc fortification in food or zinc supplementation.(7) Zinc supplementation is also commonly used to treat diarrhea and pneumonia in children.(8) Supplementing with zinc has the potential to notably boost children's appetite and improve their nutritional status.(9) If in conditions that the average intake of zinc from food alone is higher than the minimum recommendation, zinc supplement users, especially children, are at risk of excessive zinc intake. Excessive zinc intake is reported to occur in preschool children, and this is ignored by their parents, so that the potential dangers of zinc overdose are lack concern to them.(10)

Several studies have revealed the role of zinc in cognitive function, but the underlying mechanisms are still unclear. In general, studies on cognitive function in the nervous system is focusing on cell signaling that influences long-term potentiation (LTP). Synapse plasticity, which is reflected by LTP, is considered to be the basis for the formation of cognitive function.(11) Brain derived neurotrophic factor (BDNF) is a member of neurotrophins that is essential in synapse plasticity, as well as cognitive function.(12) Interestingly, BDNF signaling pathway through its receptor tropomyosin receptor kinase B (TrkB) is modulated by synaptic zinc.(13) In addition, zinc also facilitates the maturation of BDNF from its precursor proBDNF, through the activation of matrix metalloproteinase (MMP) which depends on the presence of zinc.(14) MMP is an extracellular protease that cleaves proBDNF to mature BDNF.(15) Zinc which is a natural ligand for G-protein coupled receptor 39 (GPR39), can produce a signaling cascade that activates transcription of genes that depend on cAMP response element binding protein (CREB), that subsequently promotes BDNF upregulation. Zinc intervention in chronic mild stress rats demonstrated an increase of BDNF expression in hippocampus.(16)

Several studies have evaluated the effect of maternal zinc diet on BDNF expression in brain's offspring. A previous study found that rats fed a zinc-deficient diet had offsprings with lower brain BDNF levels compared to controls.(17) Another study also showed BDNF protein in the hippocampus significantly decreased in rats on a zinc-deficient diet, and postnatal zinc supplementation after the lactation period was able to increase BDNF levels. However, it was still significantly lower than the control group.(18) In contrast, there was a study demonstrated an unexpected decrease of Zn and BDNF levels in hippocampus after high dose of Zn supplementation in 21-day-old mice for 3 months, which was followed by memory deficits and BDNF signaling disruption.(19) However, studies on the effect of maternal zinc diet on BDNF expression of brain's offspring is still limited, in addition the results of existing research are still contradictory. Furthermore, the impact of Zn intake in mother's diet on expression of CREB in offspring has not been widely studied yet. Thus, this study was conducted to analyze the impact of maternal zinc diets in offspring's cognitive function through the expression level of BDNF and CREB in their offspring's hippocampus.

Methods

Animal Model, Diets, and Sample Collection

This research was an *in vivo* experiment using Sprague Dawley rats. The protocol of this research was approved by Health Research Ethics Committee, Faculty of Medicine Universitas Indonesia (No. KET-638/UN2.F1/ETIK/PPM.00.02/2023). Ten female Sprague Dawley rats, weighing 130-150 g and 8 weeks old, were kept in a room with a temperature of 22±2°C on a daily 12-hour light-dark cycle with ad libitum access to food and water. The acclimatization period was implemented for 14 days with 20 g/day standard diet. Standard diet was made based on AIN-93M diet formulated for maintenance of adult rodents. Five 12-week-old male rats were also kept with *ad libitum* access to food and water. On the 14th day of acclimatization, mating was carried out with 1 male and 2 females in one cage. The first day of gestation begins to be counted when a vaginal plug or sperm plug was found. After that, male and female rats were separated for further treatment.

Pregnant rats were randomly divided into five groups, each group contained two rats that were fed with different zinc content (Figure 1). The feed was given at 20 g/day, every day the remaining feed was weighed to get the average value of intake per day. The zinc content in the feed for each group was described as follows: group zinc-deficient (group D) was given feed containing 2.67 mg/100 g feed Zn, starting from the first day of gestation to the 22nd day of lactation; group zinc-restricted (group R) was given feed containing 3.75 mg/100 g feed Zn, starting from the

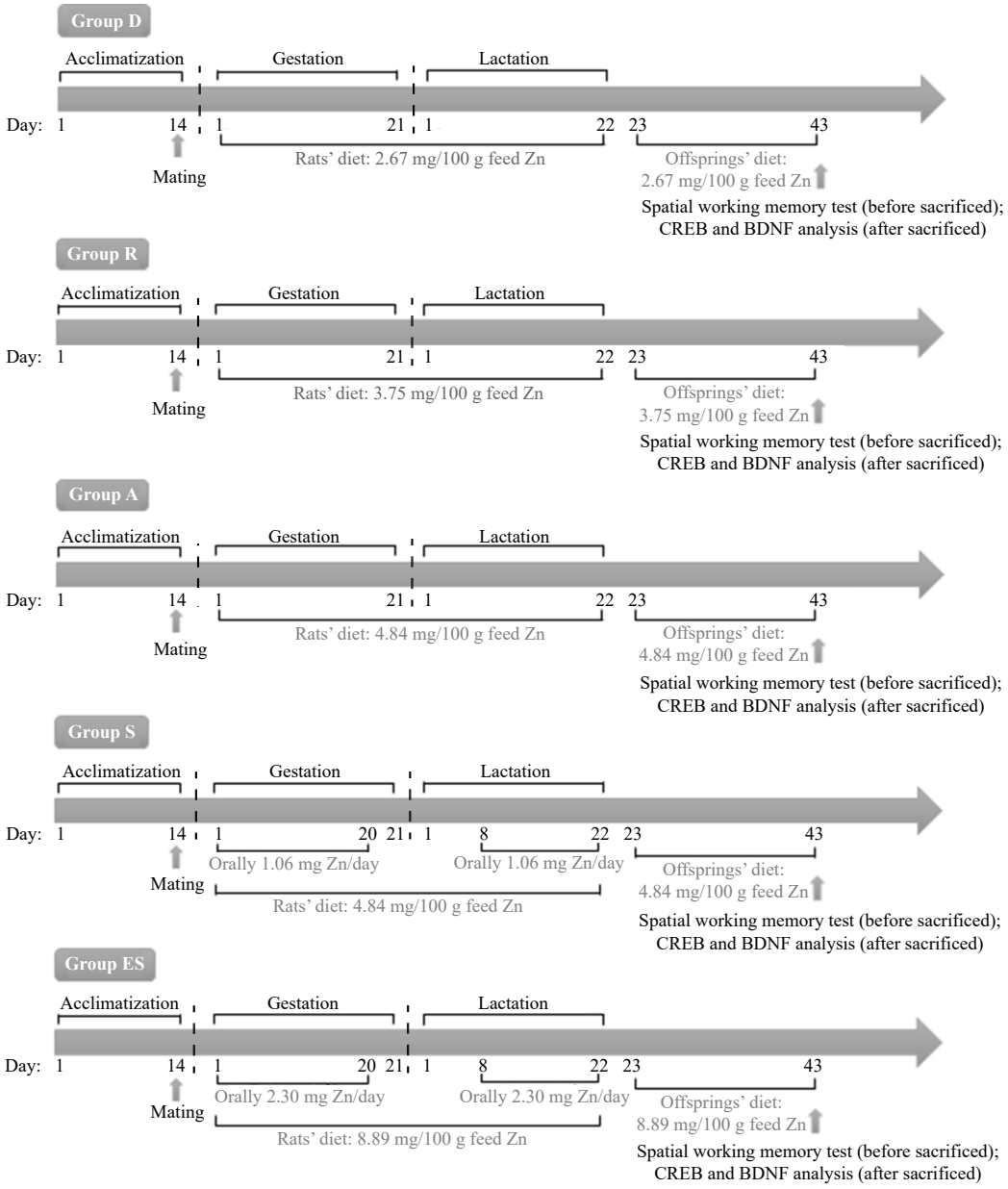

Figure 1. Diagram profile demonstrating research flow of rats and pups in each group that were fed with different Zn diet. D: zinc-deficient; R: zinc-restricted; A: zinc-adequate; S: zinc-supplemented; ES: excess zinc-supplemented.

first day of gestation to 22nd day of lactation; group zinc-adequate (group A) was given feed containing 4.84 mg/100 g feed Zn, starting from the first day of gestation to 22nd day of lactation; group zinc-supplemented (group S) was given feed containing 4.84 mg/100 g feed Zn, starting from first day of gestation to 22nd day of lactation, and also orally given 1.06 mg Zn/day during gestation (1st to 20th day) and lactation (8th to 22th day); and group excess zinc-supplemented (group ES) was given feed containing 8.89 mg/100g feed Zn, starting from the first day of gestation to 22nd day of lactation, and also orally given 2.3 mg Zn/day during gestation (1st to 20th day) and lactation (8th to 22th day). Atomic absorption spectrometry (AAS) analysis was carried out to determine the Zn content for each kind of diet. After lactation period finished (23rd postnatal day), five offsprings with similar weights were selected from each group, then separated from its mother and fed the same feed as its mother by group, until they were 43 days old. Offspring groups had *ad libitum* access to food and water. Dietary intake was measured daily, after that spatial working memory was measured, then they were sacrificed, and the brain was collected for further analysis. Rats aged 43 days were anesthetized using intraperitoneal 100 mg/kgBW ketamine. The posterior part of neck was cut towards the

face, then the cranium was split carefully in order not to damage the brain. The brain was removed from the skull bluntly, then placed directly on a petri dish in an ice box. The brain was rinsed with 0.9% NaCl to remove blood residues. The cerebellum and frontal lobe were cut using a scalpel knife, the left and right hemispheres were separated. From one hemisphere, the midbrain and colliculi were removed to reveal the hippocampal area. Hippocampus was released from cortex, then put into a storage tube and stored in a refrigerator at -80°C until further examination.

Spatial Working Memory Assessment

The evaluation of spatial working memory through the Y-maze test was conducted when the offspring rats reached 43 days of age, prior to their sacrifice (Figure 2). Rats generally prefer to move towards a new arm rather than an arm they have already passed. The Y-maze test was carried out in a closed and quiet room, using a stopwatch. The room lights were turned on dimly to reduce anxiety. Rats were allowed to explore the three arms of Y-maze freely. If a rat climbs out of the wall, it will immediately return to the arm it left behind. The arms are marked with the letters A, B, and C. Between sessions, Y maze was cleaned using 75% alcohol and allowed to dry to prevent odor cues. The number of arm entries was recorded using a video camera. An entry was recorded if the mouse enters an arm of the maze with all four paws. Spontaneous alternation was declared successful if rat enters the three different arms sequentially, calculated from the set of overlapping triples. Spontaneous alternations (%) are calculated from the number of correct entries in 3 different arms (ABC) divided by the number of possible alternations (total number of arm entries minus 2). Rats with fewer than 8 arm entries during a 10-minute trial were excluded from analysis.

Figure 2. Assessment of spatial working memory using Y-maze test was carried out at age of 43 days before offspring rats were sacrificed.

CREB Levels Measurement

CREB protein levels were measured using Rat CREB ELISA Kit (Cat. No. ID-ER3685; Indolisa, Jakarta, Indonesia), and the measurement was carried out according to the kit protocol. The microplates provided in the kit are precoated with specific antibodies to rat CREB protein. Standards were made from stock with graded concentrations, 240 pg/mL; 120 pg/mL; 60 pg/mL; 30 pg/mL; 15 pg/mL; and 0 pg/mL as blank. Fifty µL of standard solution or sample was added to the wells, then incubated for 30 minutes at 37°C. After wells washed, standards and samples were reacted with 50 µL of HRP-Streptavidin conjugate for 30 minutes at 37°C. Next, the wells were washed again, after which the standards and samples were reacted with 100 µL of TMB-substrate for 10 minutes in a dark room. Finally, 50 µL of stop solution was added, then optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The CREB concentration was determined based on the standard curve and multiplied by the number of dilutions. The CREB level (pg/mL) obtained from the ELISA results was divided by the total protein homogenate level (mg/mL), so that the unit became pg CREB per mg protein.

Quantification of *BDNF* Expression

Total RNA was isolated and purified from hippocampus using Quick RNA Miniprep Plus Kit (Cat. No. R1058; Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. PCR was carried out using SensiFAST SYBR® No ROX Real Time PCR kit (Cat. No. BIO-72005; Bioline, Nottingham, UK), following the working principle of One Step qRT-PCR. Before PCR running, concentration of RNA from each sample was equalized to 50 ng/µL. All reactions were performed in duplicates. The specific primers for *BDNF* and housekeeping gene *GAPDH* were designed based on literature studies from several previous publications. Screening for the characteristics of these primers was carried out using the National Library of Medicine Basic Local Alignment Search Tool (NIH-BLAST). Primers for *BDNF* were Forward 5'–AAGGACGCGGACTTGTACAC–3' and Reverse 5'–CGCTAATACTGTCACACACGC–3'. Primers for GAPDH were Forward 5'–TCAAGAAGGTGGTGAAGCAG– 3' and Reverse 5' –AGGTGGAAGAATGGGAGTTG– 3'. Livak formula was deliberated for relative quantification of the target gene.

Statistical Analysis

Data were analyzed using normality and homogeneity tests. Statistical analysis was performed by One Way ANOVA

or Kruskal Wallis hypothesis test. Descriptive analysis of each parameter was displayed in mean (±SD) or median (min-max). Post hoc test was used to assess any significant differences in the results. A *p*-value of 0.05 for the various outcomes was considered statistically significant.

Results

Excessive Zinc Supplementation Induced the Lowest Alteration Score

Statistical analysis was conducted using the Kruskal-Wallis test, followed by post hoc Mann-Whitney tests to further elucidate the observed differences between groups (Figure 3). The results indicate a significant discrepancy in alternation scores among the groups, although it's noteworthy that this difference was not observed between group R and group S (p=0.690) . Delving deeper into the specific findings, group R exhibited an alternation score with a median value of 79.2%, with a range spanning from 72.7% to 82.6%. Remarkably, this median value closely mirrored that of group S, which stood at 77.8% (ranging from 71.4% to 83.3%). This parity in scores between these two groups suggests a similarity in performance despite potential differences in other variables or conditions. However, the other groups showcased more pronounced distinctions in their alternation scores. Group ES emerged with the lowest median alternation score, clocking in at 63.0% (with a range from 57.9% to 66.7%). Following closely, group D demonstrated a median score of 66.7% (ranging from 66.7% to 72.7%). In stark contrast, group A boasted the highest median alternation score, soaring to 90.5% (with a range spanning from 83.3% to 100.0%).

CREB Levels Varied Notably After Adequate Zinc Intake and Excessive Zinc Supplementation

Based on the experimental data, it was evident that there exists a notable variation in the levels of CREB protein across the different treatment groups. Among these groups, group A stands out with the highest mean CREB protein level, quantified at 17.023±1.562 pg/mg protein (Figure 4). Conversely, group ES exhibits the lowest CREB concentration, recording an average value of 13.425±1.082 pg/mg protein. Following closely behind, group D presents the second lowest mean CREB level, measured at 15.808±1.463 pg/mg protein. Interestingly, group R and group S display almost indistinguishable mean CREB levels, with respective measurements of 16.966±2.312 pg/mg protein and 16.497±2.052 pg/mg protein. The statistical

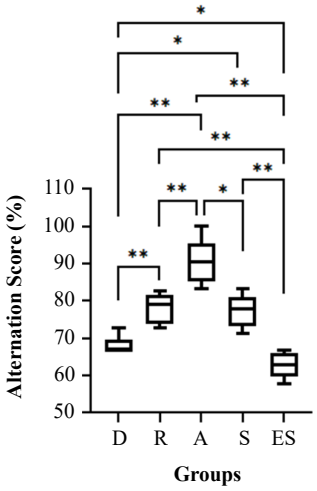

Figure 3. Comparison graph of alternation scores (spatial working memory) in offspring rats. The graph is the result of Kruskal Wallis nonparametric test with post hoc Mann-Whitney. *p≤0.05; **p≤0.01.

analysis conducted, employing One-Way ANOVA with post hoc Tukey testing, further elucidates the significance of these differences in CREB levels among the various treatment groups. Specifically, significant disparities are observed between group R and group ES (p<0.05) as well as between group A and group ES (p<0.05). However, no statistically significant distinctions in CREB levels are detected between group D and group R (p=0.831), group D and group A (p=0.806), group D and group S (p=0.970), group D and group ES (p=0.237), group R and group A (p=1.000), group R and group S (p=0.993), group A and group S (p=0.989), and group S and group ES (p=0.077).

Adequate Zinc Intake Resulted in The Highest *BDNF* Expression

According to the findings from RNA purification, the assessment involved the determination of the total

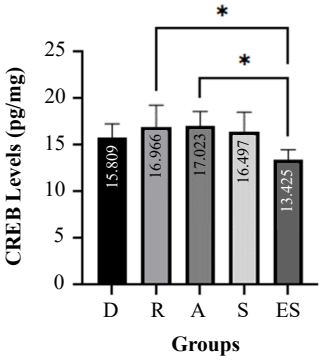

Figure 4. Comparison graph of CREB protein levels from hippocampus of offspring rats. The graph is the result of One-Way ANOVA parametric test with post hoc Tukey. *p≤0.05.

RNA concentration and purity for each sample (Table 1). Subsequently, the relative expression of *BDNF* was computed using the Livak formula, leveraging the cycle threshold value of each sample with *GAPDH* serving as the reference gene. This comprehensive analysis unveiled intriguing insights: notably, group A demonstrated the highest average relative mRNA expression of *BDNF*, recording at 2.154±0.515, whereas group ES exhibited the least expression, with a mean of 0.910±0.438 (Figure 5). Following closely, group D displayed the second lowest mean relative mRNA expression of *BDNF*, standing at 1.047±0.335. Meanwhile, group R and group S presented mean relative mRNA expressions of *BDNF* at 1.295±0.351 and 1.621±0.361, respectively. Interestingly, despite group R and group S demonstrating lower mean relative expressions compared to group A, they still showcased superior expressions in contrast to group D and group ES. Further statistical analysis, employing the One-Way ANOVA with post hoc Tukey testing, uncovered significant differences in the relative mRNA expression of BDNF across the experimental groups. Specifically, notable disparities were observed between group D and group A ($p<0.01$), group R and group A ($p<0.05$), as well as group A and group ES ($p<0.001$). These findings provide valuable insights into the relative mRNA expression patterns of *BDNF* across different experimental conditions.

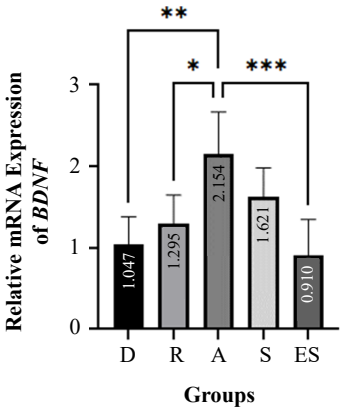

Figure 5. Comparison graph of relative mRNA expression of *BDNF* from offspring rats' hippocampus. The graph is the result of One-Way ANOVA parametric test with post hoc Tukey. *$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$.

Discussion

In current study, group D had the second-lowest alternation score at 66.7% (66.7-72.7), consistent with previous studies indicating learning and memory abnormalities.(20-22) The alternation scores, LTP amplitude, and CREB protein expression in hippocampus of Zn deficient rats were reported to be significantly lower than control group.(21) Learning deficits due to zinc deficiency involve changes in crucial signaling molecules for long-term potentiation, with decreased levels of calmodulin (CaM), CaMKII, and CREB in hippocampus of zinc-deficient mice.(22)

The group ES, given the highest Zn level food, scored the lowest alternation rate (median: 63.0%, range: 57.9-66.7%), while the group A scored highest (median: 90.5%, range: 83.3-100.0%). A previous study using mice found that high Zn intake reduced hippocampal Zn levels, and it was associated with the reduction of synaptic Zn signaling, especially in Cornu Ammonis 3 (CA3) and the dentate gyrus. These areas are crucial for hippocampal-dependent spatial learning and memory.(19,23) Depletion of synaptic zinc in ZnT3 knock-out mice resulted in complete deficits in spatial working memory, affecting hippocampus-dependent learning.(24) In humans, Zn supplementation in malnourished infants with Zn deficiency led to diminished cognitive performance, contrary to expectations.(25)

Group A exhibited the highest average CREB protein levels, whereas group D and group ES showed a tendency towards decreased concentrations. This aligns with prior research indicating a significant 44% reduction in CREB protein levels in rat hippocampus after six weeks of Zn-deficient diet.(26) Immunohistochemical analysis revealed a significant decrease in phosphorylated CREB (p-CREB) immunoreactivity in hippocampus of zinc-deficient rats, despite no change in total CREB levels compared to controls.(22) Measurement of CREB protein levels aims to assess molecular changes related to hippocampal long-term potentiation, with CREB being a downstream protein of pathways like CaMKII. Activated CaMKII phosphorylates CREB at Serine 133, activating gene transcription and promoting neuronal plasticity and LTP formation.(27) Downregulation of CREB was observed in

Table 1. Hippocampal RNA concentration and purity of 43-day offsprings.

Groups	Group Mean±SD	
	Concentration (ng/µL)	Purity
D (n=5)	201.8±67.335	1.805±0.090
R (n=5)	360.3±46.445	1.837±0.078
A (n=5)	233.2±68.589	1.814±0.045
S (n=5)	340.8±106.498	1.846±0.053
ES (n=5)	162.8±43.436	1.789±0.043

Alzheimer's disease (28), and its dysregulation is implicated in autism spectrum disorder (29), with low plasma CREB concentrations correlating with symptom severity (30). Zn supplementation has been beneficial in improving autism symptoms by potentially modulating CREB levels.(30)

In this study, CREB protein levels were significantly lower in the group ES compared to the groups R and A (Figure 4). Total and phosphorylated CREB levels decreased significantly in mice treated with Zn supplementation. (19) Despite CREB signaling pathway disruption, food fortification and zinc supplementation may reduce CREB levels due to nerve cell death caused by reactive oxygen species (ROS) formation. Excitotoxic stimulation during ischemia can release large amounts of glutamate and Zn, leading to Zn entering postsynaptic neurons.(31) Previous research indicates Zn exposure increases nicotinamide adenine dinucleotide phosphate (NADPH) oxidase levels in neurons and astrocytes, promoting oxidative stress.(32) This enzyme, located in membranes and cytoplasm, produces superoxide and is activated by protein kinase C.(33) The interplay between Zn and ROS is closely linked to nerve cell death, particularly in conditions involving Zn accumulation in the brain like traumatic brain injury, ischemic stroke, and hypoglycemia.(34)

Group A showed the highest *BDNF* mRNA expression, while group ES had the lowest, with a significant difference between them (Figure 5). Previous research found that high zinc supplementation reduced zinc levels in the hippocampus, leading to a deficit in synaptically released zinc in CA3 and dentate gyrus.(19) This resulted in decreased *BDNF* levels and reduced total and phosphorylated CREB levels, indicating inhibition of CREB-BDNF signaling due to hippocampal zinc deficiency.(19)

Group D had the lowest zinc diet while group A had adequate zinc. There was a significant difference in *BDNF* mRNA expression between these groups. Using rats as animal models, administering zinc for 14 days reduced glycine/NMDA receptor binding in the frontal cortex and increased 5-HT(1A) and 5-HT(2A) receptor density in the hippocampus and frontal cortex, linked to elevated *BDNF* levels. These results indicate that Zn therapy alters the glutamatergic and serotonergic systems.(35) Additionally, intraperitoneal injection of 15 mg/kg zinc hydroaspartate increased hippocampal *BDNF* mRNA expression in stress and non-stress rat models.(36) In a chronic mild stress rat model, zinc treatment increased hippocampal *BDNF* mRNA and BDNF protein levels by 17-39%.(37)

Efforts to address zinc deficiency through fortification and supplementation have increased, yet the impact of disrupted zinc balance on hippocampal function remains underexplored. This study suggests that zinc imbalance affects hippocampal function, evidenced by reduced CREB and BDNF expression in both deficient and excess zinc groups. Plausible explanations include: 1) Zinc imbalance may lead to deficient synaptically released zinc in the hippocampus, altering signaling molecules like CREB crucial for neuronal plasticity and LTP induction (19,22); 2) Excess zinc exposure could increase neuronal and astrocytic NADPH oxidase levels, causing oxidative stress and reducing CREB, potentially leading to neuronal cell death (32); 3) Zinc binding to the GPR39 receptor activates signaling pathways that upregulate BDNF expression, influencing hippocampal function (38); 4) Zinc's role as an NMDA receptor antagonist may alter *BDNF* expression indirectly by modulating receptor activity (39); and 5) Additionally, zinc induces MMP activity, crucial for processing pro-BDNF to its mature form, promoting synaptic plasticity and neuronal survival (40).

In summation , the multifaceted interplay between zinc levels and hippocampal function underscores the intricate mechanisms governing neuronal homeostasis and synaptic plasticity within the brain. More investigation is necessary to explore zinc involvement on the mechanism of the CREB-BDNF interaction so that the molecular processes underlying cognition can one day be fully understood.

Conclusion

In conclusion, this study demonstrated that maternal and post lactation zinc diets in deficient and excess doses impaired spatial working memory and decreased CREB and BDNF levels. This study suggests that zinc dyshomeostasis induces disruption of CREB/BDNF signaling, which further impacts on learning and memory.

Acknowledgments

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Authors Contribution

All authors participated in developing the research idea, interpreting the results, and preparing the manuscript. NDT conducted data acquisition and analysis. NAW, NM, and ARP contributed to critically revising the manuscript.

References

1. Narváez-Caicedo C, Moreano G, Sandoval BA, Jara-Palacios MÁ. Zinc deficiency among lactating mothers from a Peri-Urban Community of the Ecuadorian Andean Region: an initial approach to the need of zinc supplementation. Nutrients. 2018; 10(7): 869. doi: 10.3390/nu10070869.
2. Caulfield LE, Black RE. Zinc deficiency. In: Ezzati M, Lopez AD, Rodgers A, Murray CJL, editors. Comparative Quantification of Health Risks: Global and Regional Burden of Diseases Attributable to Selected Major Risks. Geneva: World Health Organization; 2004. p.257-79.
3. Fernandes FS, de Souza AS, do Carmo M, Boaventura GT. Maternal intake of flaxseed-based diet (Linum usitatissimum) on hippocampus fatty acid profile: implications for growth, locomotor activity and spatial memory. Nutrition. 2011; 27(10): 1040-7.
4. Shah D, Sachdev HP. Zinc deficiency in pregnancy and fetal outcome. Nutr Rev. 2006; 64(1): 15-30.
5. Gao HL, Zheng W, Xin N, Chi ZH, Wang ZY, Chen J, *et al.* Zinc deficiency reduces neurogenesis accompanied by neuronal apoptosis through caspase-dependent and -independent signaling pathways. Neurotox Res. 2009; 16(4): 416-25.
6. Guidolin D, Polato P, Venturin G, Zanotti A, Mocchegiani E, *et al.* Correlation between zinc level in hippocampal mossy fibers and spatial memory in aged rats. Ann NY Acad Sci. 1992; 673: 187-93.
7. Yu SM, Kogan MD, Gergen P. Vitamin-mineral supplement use among preschool children in the United States. Pediatrics. 1997; 100(5): E4. doi: 10.1542/peds.100.5.e4.
8. Brown KH, Peerson JM, Baker SK, Hess SY. Preventive zinc supplementation among infants, preschoolers, and older prepubertal children. Food Nutr Bull. 2009; 30(1): 12-40.
9. Kusumastuti AC, Ardiaria M, Hendrianingtyas M. Effect of zinc and iron supplementation on appetite, nutritional status, and intelligence quotient in young children. Indones Biomed J. 2018; 10(2): 133-9.
10. Huybrechts I, Maes L, Vereecken C, De Keyzer W, De Bacquer D, *et al.* High dietary supplement intakes among Flemish preschoolers. Appetite. 2010; 54(2): 340-5.
11. Malik R, Chattarji S. Enhanced intrinsic excitability and EPSP-spike coupling accompany enriched environment-induced facilitation of LTP in hippocampal CA1 pyramidal neurons. J Neurophysiol. 2012; 107(5): 1366-78.
12. Bramham CR, Messaoudi E. BDNF function in adult synaptic plasticity: The synaptic consolidation hypothesis. Prog Neurobiol. 2005; 76(2): 99-125.
13. Hwang JJ, Park MH, Choi SY, Koh JY. Activation of the Trk signaling pathway by extracellular zinc: Role of metalloproteinases. J Biol Chem. 2005; 280(12): 11995-2001.
14. Yoo MH, Kim TY, Yoon YH and Koh JY. Autism phenotypes in ZnT3 null mice: involvement of zinc dyshomeostasis, MMP-9 activation and BDNF upregulation. Nat Sci Rep. 2016; 6: 28548. doi: 10.1038/srep28548.
15. Cunha C, Brambilla R, Thomas KL. A simple role for BDNF in learning and memory? Front Mol Neurosci. 2010; 3: 1. doi: 10.3389/neuro.02.001.2010.
16. Sowa-Kucma M, Legutko B, Szewczyk B, Novak K, Znojek P, Poleszak E, *et al.* Antidepressant-like activity of zinc: further behavioral and molecular evidence. J Neural Transm. 2008; 115; 1621-28.
17. Chowanadisai W, Kelleher SL, Lonnerdal B. Maternal zinc deficiency reduces NMDA receptor expression in neonatal rat brain, which persists into early adulthood. J Neurochem. 2005; 94: 510-9.
18. Yu X, Ren T, Yu X. Disruption of calmodulin-dependent protein kinase II α/brain-derived neurotrophic factor (α-CaMKII/BDNF) signalling is associated with zinc deficiency-induced impairments in cognitive and synaptic plasticity. Br J Nutr. 2013; 110(12): 2194-200.
19. Yang Y, Jing XP, Zhang SP, Gu RX, Tang FX, *et al.* High dose zinc supplementation induces hippocampal zinc deficiency and memory impairment with inhibition of BDNF signaling. PLoS ONE. 2013; 8(1): e55384. doi: 10.1371/journal.pone.0055384.
20. Yu X, Jin L, Zhang X, Yu X. Effects of maternal mild zinc deficiency and zinc supplementation in offspring on spatial memory and hippocampal neuronal ultrastructural changes. Nutrition. 2013; 29(2): 457-61.
21. Jiang YG, Fang HY, Pang W, Liu J, Lu H, Ma Q, Fang HT. Depressed hippocampal MEK/ERK phosphorylation correlates with impaired cognitive and synaptic function in zinc-deficient rats. Nutr Neurosci. 2011; 14(2): 45-50.
22. Gao HL, Xu H, Xin N, Zheng W, Chi ZH, Wang ZY. Disruption of the CaMKII/CREB signaling is associated with zinc deficiency-induced learning and memory impairments. Neurotox Res. 2011; 19(4): 584-91.
23. Yoshida K, Gi M, Fujioka M, Teramoto I, Wanibuchi H. Long-term administration of excess zinc impairs learning and memory in aged mice. J Toxicol Sci. 2019; 44(10): 681-91.
24. Sindreu C, Palmiter RD, Storm DR. Zinc transporter ZnT-3 regulates presynaptic Erk1/2 signaling and hippocampus-dependent memory. Proc Natl Acad Sci USA. 2011; 108(8): 3366-70.
25. Hamadani JD, Fuchs GJ, Osendarp SJ, Huda SN, Grantham-McGregor SM. Zinc supplementation during pregnancy and effects on mental development and behaviour of infants: a follow-up study. Lancet. 2002; 360(9329): 290-4.
26. Doboszewska U, Szewczyk B, Sowa-Kućma M, Młyniec K, Nowak G. The disruption of CREB/BDNF/TrkB signaling. Dynamic changes induced by zinc deficiency. Pharmacol Rep. 2013; 65 (Suppl 1): 119-20.
27. Tropea TF, Kosofsky BE, Rajadhyaksha AM. Enhanced CREB and DARPP-32 phosphorylation in the nucleus accumbens and CREB, ERK, and GluR1 phosphorylation in the dorsal hippocampus is associated with cocaine-conditioned place preference behavior. J Neurochem. 2008; 106(4): 1780-90.
28. Saura CA, Valero J. The role of CREB signaling in Alzheimer's disease and other cognitive disorders. Rev Neurosci. 2011; 22(2): 153-69.
29. Kasarpalkar NJ, Kothari ST, Dave UP. Brain-derived neurotrophic factor in children with autism spectrum disorder. Ann Neurosci. 2014; 21(4): 129-33.
30. Russo AJ, Mensah A, Bowman J. Decreased phosphorylated CREB and AKT in individuals with autism normalized after zinc therapy. Acad J Ped Neonatol. 2017; 5(3): 57-60.
31. Li Y, Hough CJ, Suh SW, Sarvey JM, Frederickson CJ. Rapid

translocation of Zn(2+) from presynaptic terminals into postsynaptic hippocampal neurons after physiological stimulation. J Neurophysiol. 2001; 86(5): 2597-604.

32. Noh KM, Koh JY. Induction and activation by zinc of NADPH oxidase in cultured cortical neurons and astrocytes. J Neurosci. 2000; 20(23): RC111. doi: 10.1523/JNEUROSCI.20-23-j0001.2000.
33. Chen F, Yu Y, Haigh S, Johnson J, Lucas R, Stepp DW, Fulton DJ. Regulation of NADPH oxidase 5 by protein kinase C isoforms. PLoS One. 2014; 9(2): e88405. doi: 10.1371/journal.pone.0088405.
34. Marreiro DD, Cruz KJ, Morais JB, Beserra JB, Severo JS, de Oliveira AR. Zinc and oxidative stress: Current mechanisms. Antioxidants. 2017; 6(2): 24. doi: 10.3390/antiox6020024.
35. Cichy A, Sowa-Kućma M, Legutko B, Pomierny-Chamioło L, Siwek A, Piotrowska A, *et al.* Zinc-induced adaptive changes in NMDA/glutamatergic and serotonergic receptors. Pharmacol Rep. 2009; 61(6): 1184-91.
36. Cieślik K, Sowa-Kućma M, Ossowska G, Legutko B, Wolak M, Opoka W, *et al.* Chronic unpredictable stress-induced reduction in the hippocampal brain-derived neurotrophic factor (BDNF) gene expression is antagonized by zinc treatment. Pharmacol Rep. 2011; 63(2): 537-43.
37. Sowa-Kućma M, Legutko B, Szewczyk B, Novak K, Znojek P, Poleszak E, *et al.* Antidepressant-like activity of zinc: further behavioral and molecular evidence. J Neural Transm. 2008; 115(12): 1621-8.
38. Mlyniec K. Zinc in the glutamatergic theory of depression. Curr Neuropharmacol. 2015; 13(4): 505-13.
39. Nowak G, Szewczyk B. Mechanisms contributing to antidepressant zinc actions. Pol J Pharmacol. 2002; 54(6): 587-92.
40. Hwang JJ, Park MH, Choi SY, Koh JY. Activation of the Trk signaling pathway by extracellular zinc. Role of metalloproteinases. J Biol Chem. 2005; 280(12): 11995-2001.