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CHRONIC BUT NOT ACUTE ANTIDEPRESSANT TREATMENT ALTERS SERUM ZINC/COPPER RATIO UNDER PATHOLOGICAL/ZINC-DEFICIENT CONDITIONS IN MICE

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Depression is the leading psychiatric disorder with a high risk of morbidity and mortality. Clinical studies report lower serum zinc in depressed patients, suggesting a strong link between zinc and mood disorders. Also copper as an antagonistic element to zinc seems to play a role in depression, where elevated concentration is observed. In the present study we investigated serum copper and zinc concentration after acute or chronic antidepressant (AD) treatment under pathological/zinc-deficient conditions. Zinc deficiency in mice was induced by a special diet administered for 6 weeks (zinc adequate diet – ZnA, contains 33.5 mgZn/kg; zinc deficient diet – ZnD, contains 0.2 mgZn/kg). Animals received acute or chronically saline (control), imipramine, escitalopram, reboxetine or bupropion. To evaluate changes in serum copper and zinc concentrations the total reflection X-ray fluorescence (TXRF) and flame atomic absorption spectrometry (FAAS) was performed. In ZnD animals serum zinc level was reduced after acute ADs treatment (similarly to vehicle treatment), however, as demonstrated in the previous study after chronic ADs administration no differences between both ZnA and ZnD groups were observed. Acute ADs in ZnD animals caused different changes in serum copper concentration with no changes after chronic ADs treatment. The calculated serum Zn/Cu ratio is reduced in ZnD animals (compared to ZnA subjects) treated with saline (acutely or chronically) and in animals treated acutely with ADs. However, chronic treatment with ADs normalized (by escitalopram, reboxetine or bupropion) or increased (by imipramine) this Zn/Cu ratio. Observed in this study normalization of serum Zn/Cu ratio in depression-like conditions by chronic (but not acute) antidepressants suggest that this ratio may be consider as a marker of depression or treatment efficacy.

Key words: *copper, zinc/copper ratio, zinc deficiency, depression, serum, antidepressant, proinflammatory cytokines, oxidative stress*

INTRODUCTION

Depression is the leading disorder in relation to morbidity and mortality. Commonly used antidepressants show delayed therapeutic effects and generate many side effects (1). Moreover, about 30% of depressed patients do not respond to antidepressant therapy (2). The end of the last century brought a new insight into the pathophysiology of depression. An excessive glutamate release seems to be a cause of depressive symptoms (3-5) and antagonists at the glutamatergic NMDA receptor seems to exhibit antidepressant properties (3). Zinc as well as copper inhibit NMDA-induced currents (6, 7) and modulate glutamatergic neurotransmission. A single dose of zinc administration was found to produce antidepressant and anxiolytic effect (8). Zinc and copper are two important trace elements required for appropriate body functions. Adequate zinc intake in adults ranges of 11 mg/day for male and 8 mg/day for female (9). The recommended dietary allowance (RDA) of copper for men and women is 0.9 mg/day. Daily requirement for those elements increase during pregnancy and lactation (1–2 mgZn/day, 1–1.3 mg Cu/day) (9). Deficiency as well as excess amounts of zinc and copper lead to neurotransmission impairment, immune dysfunction and oxidative stress (10, 11), which causes psychiatric disability and depression. Malavolta *et al*. (12) showed a link between elevated inflammatory markers and increased Cu and decreased Zn levels. The authors proposed Zn/Cu ratio as an important clinical inflammatory-nutritional biomarker. Moreover, they suggest the ratio of copper and zinc as a significant predictor of ”all-cause mortality” in people over 70 years of age (12). Clinical studies showed that immunological changes such as elevated proinflammatory cytokines accompanied major depression (13-15). Maes *et al*. (15) explained that lower serum zinc observed in depressed patients may be due to enhanced sequestration by metallothioneins in the liver, which may be a consequence of increased interleukin 6 (IL-6) production. In other study of Maes *et al*. (14), serum zinc and copper was analyzed in treatment resistant forms of depression. Non-responders to commonly used antidepressant therapy

showed lower baseline serum zinc than healthy controls, which negatively correlated with the CD4+/CD8+ T-cell ratio and positively correlated with total serum protein, serum albumin, and transferrin. There were no changes after antidepressant treatment. Opposite to zinc serum copper was found to be significantly reduced after antidepressant treatment (14).

Based on the data from preclinical and clinical studies on the involvement of zinc and copper in depressive disorders, in the present study we investigated the serum concentrations of those elements after acute or chronic antidepressant treatment under zinc-deficient conditions, which is proposed as a model of depression.

MATERIALS AND METHODS

Animals and diet schedule

All of the procedures were conducted according to the National Institute of Health Animal Care and Use Committee guidelines, which were approved by the Ethical Committee of the Jagiellonian University Medical College in Cracow.

Three-week-old male CD-1 Swiss mice (~16 g) were housed under standard laboratory conditions with a natural day-night cycle, a temperature of 22±2°C and the humidity at 55±5% as well as access to food and water *ad libitum*. Zinc adequate (ZnA, 33.5 mg Zn/kg) or zinc deficient diet (ZnD, 0.2mg Zn/kg) purchased from MP Biomedicals (France), were administered for 6 weeks.

Drug treatment

According to Mlyniec *et al*. (16) mice received andidepressants (i.p.) with diverse mechanism of action: (1) imipramine (tricyclic antidepressant, 30 mg/kg, Sigma-Aldrich, USA), (2) escitalopram (selective serotonin reuptake inhibitor, 4 mg/kg, Lundbeck, Denmark), (3) reboxetine (selective noradrenaline reuptake inhibitor, 10 mg/kg, Ascent, UK), (4) bupropion (selective noradrenaline-dopamine reuptake inhibitor, 15 mg/kg, Sigma-Aldrich, USA), or saline (0.9% NaCl) for two weeks or in a single dose. The dosage of drugs we used in this study were determined previously as the lowest active dose in the forced swim test. The experiments were performed examining each of drug separately in ZnA and ZnD animals. The schedule of diet and drug administration was as follows:

- Six-week zinc adequate or zinc deficient diet + acute drug treatment on the last day of diet administration.
- Four-week zinc adequate or zinc deficient diet + additional 2-week zinc adequate or zinc deficient diet and 2-week drug treatment.

Zinc and copper assay

All animals were killed by rapid decapitation, 24 hours after acute or the last drug treatment, the blood was collected. The serum was separated by centrifugation.

The total reflection X-ray fluorescence

As described by (15), the serum samples of mice were irradiated by a primary X-ray beam. The TXRF method is dedicated to the liquid samples and is suitable for trace analysis. The 2–10 µl of the sample is deposited on the optically flat surface of the reflector. The quantitative analysis is performed after the addition of the internal standard. To the 20 µl of the serum sample, galium as an internal standard was added to achieve the final concentration 5 mg/L. For the measurements, the TXRF spectrometer Nanohunter (Rigaku) was used. The single measurement time was 2000 s and the Mo X-ray tube was used (50 kV, O.8 mA).

Flame atomic absorption spectrometry

According to (16), zinc and copper levels were determined in serum. The thawed samples were mixed and then analyzed directly by the atomic absorption spectrometry (AAS) method. In some instances (the smallest volume), the electrothermal technique (ET AAS) was used, while for higher volume, the flame technique (F AAS) was applied. Precision of the measurements was less than 7% (RSD). The accuracy of measurements was checked *via* the F AAS and voltammetry (ASV) methods (means of comparative analysis of digested sample). Less than 2% was the difference between the obtained results. PerkinElmer Model 3110 (USA) a spectrometer was used to determine the Zn and Cu concentrations. To perform electrothermal analysis a PerkinElmer HGA 600 instrument was used.

Data analysis

The obtained data was evaluated by the Student *t*-test (GraphPad Prism software, CA). All the results are presented as the mean of Zn/Cu ratio ± S.E.M., $P<0.05$ was considered to be statistically significant.

RESULTS

Effect on serum zinc and copper level

Both acute or chronic NaCl (saline) administration to zinc-deficient mice causes a significant reduction in serum zinc concentration (by 68% and 34%, respectively), but not in copper concentration when compare to zinc-adequate animals (*Table 1*). Acute administration of all tested antidepressants to zinc-deficient mice did not influence on this zinc deficiency-induced reduction, however, chronic treatment normalized this effect (*Table 1*). From the other hand, serum copper level in zinc-deficiency is increased by acute but not by chronic administration of imipramine, escitalopram and reboxetine when compared to zinc-adequate animals (*Table 1*). Whereas, both acute and chronic treatment with bupropion did not influence this parameter (*Table 1*).

Effect on serum zinc/copper (Zn/Cu) ratio

Six-week zinc-deficient diet causes a significant decreases in serum zinc/copper ratio when compare to zinc-adequate diet after acute [$t(10) = 7.632$; $P<0.0001$] or chronic [$t(8) = 2.446$; $P<0.0402$] saline administration (*Fig. 1A*).

Acute imipramine administration causes a significant decrease in serum Zn/Cu ratio in comparison to zinc-adequate diet [$t(10) = 4.230$; $P = 0.0017$], while chronic imipramine treatment causes a significant increase in Zn/Cu ratio [$t(12) = 2.597$; $P = 0.0234$] (*Fig. 1B*).

Acute escitalopram treatment in zinc deficiency significantly decreases levels of serum Zn/Cu ratio [$t(10) = 22.94$; $P<0.0001$]. There are no changes after chronic escitalopram administration [$t(10) = 0.5271$; $P<0.6096$] (*Fig. 1C*).

Acute reboxetine treatment causes a significant decrease in serum Zn/Cu ratio [$t(10) = 2.432$; $P = 0.0354$], while there are no changes in serum Zn/Cu ratio after chronic reboxetine administration [$t(8) = 1.338$; $P = 0.2176$] (*Fig. 1D*).

Acute bupropion treatment in zinc deficiency causes a significant decrease in Zn/Cu ratio [$t(10) = 8.010$; $P<0.0001$], while there are no changes in Zn/Cu ratio after chronic bupropion treatment [$t(11) = 1.110$; $P=0.2905$] (*Fig. 1E*).

Table 1. The effect of antidepressant treatment on serum zinc and copper concentration in zinc adequate (ZnA) and zinc deficient (ZnD) mice, measured 24 hours after acute or chronic drug administration. Results are expressed as mean ± S.E.M. of 5–7 animals per group. * P<0.05 means ZnD proper ZnA (control group). Data in italics are derived from our previous serum zinc examinations included for comparison with permission from Mlyniec *et al*. *Pharmacol Rep* 2013 (16).

Zinc	ZnA Acute ADs	ZnD Acute ADs	ZnA Chronic ADs	ZnD Chronic ADs
NaCl	1.35 ± 0.05	0.43 ± 0.10*	*1.93 ± 0.50*	*1.28 ± 0.05**
Imipramine	1.43 ± 0.08	1.05 ± 0.08*	*1.69 ± 0.11*	*1.94 ± 0.05*
Escitalopram	1.95 ± 0.02	0.69 ± 0.01*	*1.73 ± 0.10*	*1.82 ± 0.11*
Reboxetine	1.54 ± 0.09	0.80 ± 0.10*	*2.15 ± 0.18*	*1.97 ± 0.05*
Bupropion	1.36 ± 0.09	0.82 ± 0.09*	*1.86 ± 0.15*	*1.90 ± 0.10*
Copper	ZnA Acute ADs	ZnD Acute ADs	ZnA Chronic ADs	ZnD Chronic ADs
NaCl	1.17 ± 0.05	1.26 ± 0.12	0.68 ± 0.04	0.59 ± 0.04
Imipramine	1.08 ± 0.02	1.48 ± 0.15*	0.67 ± 0.02	0.62 ± 0.04
Escitalopram	1.08 ± 0.02	0.69 ± 0.01*	0.57 ± 0.03	0.56 ± 0.03
Reboxetine	1.28 ± 0.07	1.08 ± 0.04*	0.65 ± 0.08	0.56 ± 0.03
Bupropion	1.16 ± 0.05	1.16 ± 0.08	0.63 ± 0.02	0.61 ± 0.03

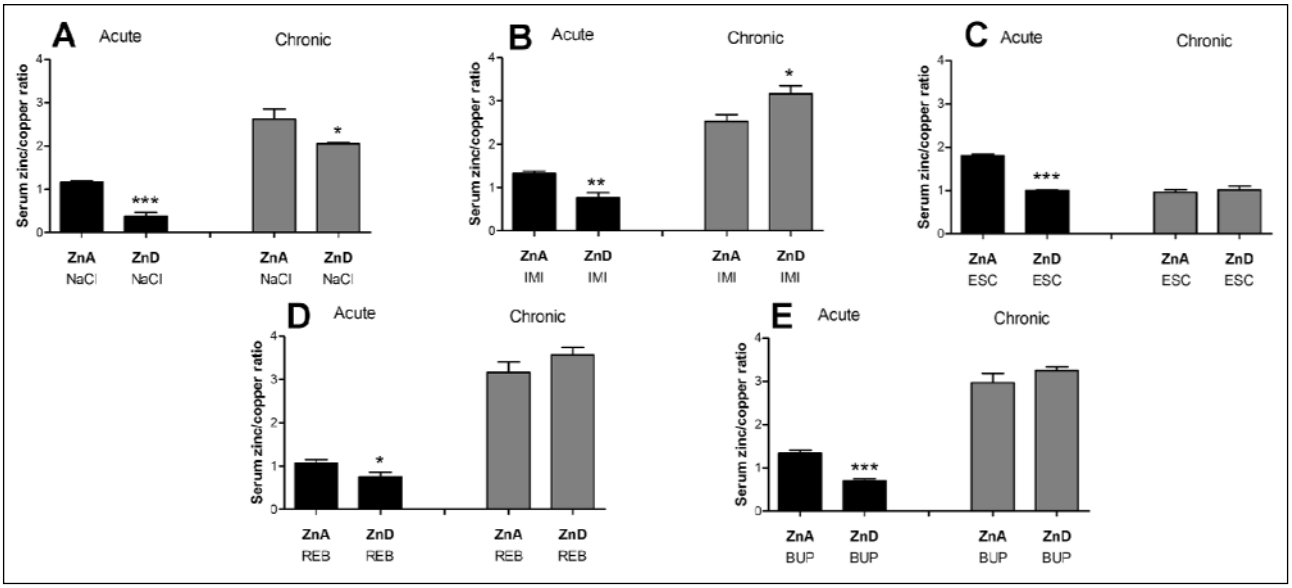

Fig. 1. The influence of acute or chronic treatment with saline (NaCl, A), imipramine (30 mg/kg, B), escitalopram (4 mg/kg, C), reboxetine (10 mg/kg, D), bupropion (15 mg/kg, E) on serum zinc/copper ratio in mice receiving 6-week zinc-adequate (ZnA) or zinc-deficient (ZnD) diet. Values are the means ± S.E.M. of 5–7 animals per group. * P<0.05; ** P<0.01; *** P<0.001 vs. proper control.

The summary of the effect of antidepressant treatment on serum Zn/Cu ratio in ZnD vs. ZnA mice is shown in *Table 2*.

DISCUSSION

Depression is one of the most frequently occuring disorder (17) The link between zinc deficiency and depression is well known (18). Ours and other studies showed behavioral abnormalities after a zinc-deficient diet in the forced swim test (19-22) and tail suspension test (20, 23). Administration of the diet low in zinc caused a significant reduction in the serum zinc level, which was normalized after chronic ADs treatment with diverse mechanism of action, such as imipramine (nonselective serotonin-noradrenaline reuptake inhibitor), escitalopram (selective serotonin reuptake inhibitor), reboxetine (selective

Table 2. Summary of the effect of antidepressant treatment on serum Zn/Cu ratio in ZnD vs. ZnA mice. Arrows indicate the increase and reduction of Zn/Cu ratio; the "-" symbol indicates no effect.

Treatment	NaCl (0.9%)	Escitalopram	Imipramine	Bupropion	Reboxetine
Acute	↓	↓	↓	↓	↓
Chronic	↓	-	↑	-	-

noradrenaline reuptake inhibitor) or bupropion (selective noradrenaline-dopamine reuptake inhibitor) (16).

Improvement in the general mood conditions in patients suffering from depression appeared after a few weeks of antidepressant treatment, not after a single dose. Since, chronic ADs treatment in animals subjected to pathological conditions (chronic unpredictable stress, zinc-deficiency) normalized pathology-induced reduction in serum zinc concentrations (16, 23), serum zinc level as well as zinc/copper ratio, presented in this study, could be a marker for effective therapy.

Chronic ADs treatment differently affects serum zinc concentration (imipramine: reduction or has no effect while citalopram increase) in "normal" rats (24, 25). Our previous study (16) demonstrated no effect of chronic antidepressant treatment on serum zinc level in mice (ZnA). This data is included for comparison in the present article (with permission from *Pharmacological Reports*). Thus, it means that ADs normalized the reduced blood zinc level (induced by pathological mechanisms), but their effect on normal physiological levels is different.

As a matter of fact serum zinc level is proposed as a potential clinical marker of depressive disorder (15, 26-28), also in treatment resistant depression. One of the hypothesis of the cause of low zinc levels in depressed patients is the sequestration of zinc by metallothioneins in the liver (10), which seems to be related to an increased activity of pro-inflammatory cytokines, such as interleukin-1 and -6 (IL-1, IL-6), often observed to be raised in depressed patients (29). Administration of fluoxetine decreases previously elevated IL-6 in depressed patients (30). This may decrease activity of HPA axis, which is involved in stress and depression (31) and reduce concentration of metallothioneins, proteins releasing zinc in case of its deficiency. Such mechanism may explain normalization of serum zinc levels after chronic ADs administration, but it needs further investigation. Recent studies indicate the involvement of the pro-inflammatory cytokines also in neurotransmitter metabolism and neural plasticity (32). Administration of zinc significantly lowers inflammation (33), and thereby may influence on neural plasticity improvement. It was found that a high-dose zinc supplementation decreases inflammatory processes in patients suffering from type-2-diabetes (34), which is often correlated to depressive symptoms (35-36). Recent years showed that there is a link between zinc and diabetes. GPR39 $Zn(2^+)$-sensing receptor seems to play an important role in that illness and was proposed as a novel potential target for diabetes (37-38). Based on this data in our previous studies we investigated the zinc receptor in terms of depression. Our first results showed possible involvement of the GPR39 zinc receptor in the pathophysiology of depressive disorders (19, 39, 40).

Depression is linked to oxidative stress (41-42). Studies by Ozturk *et al*. (43) showed the imbalance of the trace elements such as zinc and copper in oxidative stress, in which zinc levels are significantly lower whilst copper concentrations are elevated.

In the present study we observed a variety of alterations in the serum copper level induced by acute antidepressant treatment. Escitalopram and reboxetine reduced, imipramine increased, while bupropion did not alter serum copper level in ZnD group in comparison to ZnA group treated with appropriate antidepressant. On the other hand, such a comparison revealed no alterations in the serum copper level after chronic treatment with all examined antidepressants. Moreover, we observed a much higher zinc concentration compared to copper concentration after each antidepressant in a zinc-adequate diet as well as a zinc-deficient diet. This is consistent with data showing the antagonistic effects of zinc and copper. Opposite to lower zinc levels, higher copper concentrations in depressive disorder are observed (44). Also women suffering from post-partum depression show a significant increase in serum copper (45) and low level of zinc (46). Patients suffering from depression have higher serum copper levels than healthy controls (47-48), which are significantly reduced (15, 49) or not altered (48) after antidepressant treatment. Preclinical study confirmed reduced serum copper levels after chronic treatment with imipramine and citalopram (50). Analysis of essential trace elements in patients suffering from anxiety often associated to depression shows a similarity to other findings significantly decreased serum zinc and increased serum copper concentrations (51).

Besides proposed blood (serum, plasma) zinc or copper concentrations as markers of depression and/or treatment efficacy (48, 52-53) the ratio zinc/copper (Zn/Cu) might be also proposed as a useful marker. When we analyze the serum ratio Zn/Cu in clinical depression the lowered value of such ratio can be demonstrated. Namely, two articles (54-55) presented data of serum zinc and copper level (respectively) in the same groups of depressed patients and healthy volunteers (controls) and when the ratio Zn/Cu is calculated (based on these data) the ratio of 0.95 in control is reduced to 0.69 in depressed patients. Calculation of such ratio of the data from the same articles concerning animal models of depression indicate similar reduction in the ratio Zn/Cu in rats subjected to chronic severe stress (from 1.24 in control to 0.9 in stress), but not in chronic mild stress or olfactory bulbectomy models. It seems that the application to animals of more severe stress is necessary to demonstrate such reduction, which is supported by the recent findings of Zhou *et al*. (56-57) who demonstrated reduction of serum Zn/Cu ratio in rats subjected modification of Katz stress model (58).

The present study supports this issue that zinc deficiency (proposed model of depression) reduced serum Zn/Cu ratio and chronic treatment increased this reduced value (*Table 2*).

Our present study shows changes in serum zinc and copper concentrations after antidepressant treatment in zinc-deficient diet fed mice. Observed in the previous study normalization (increased) of serum zinc in pathological/depression-like conditions by chronic antidepressants with concomitant stable serum copper level (present data) suggests, that those elements may be considered as a state and trait marker (respectively) of depression (depression-like conditions).

Moreover, observed in this study normalization (or increased) of serum Zn/Cu ratio in pathological/depression-like conditions by chronic (but not acute) antidepressants suggest the

demand for future evaluation of this ratio as a marker of depression/efficacy of treatment.

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