

**БИОХИМИЯ, ГЕНЕТИКА
И МОЛЕКУЛЯРНАЯ БИОЛОГИЯ****BIOCHEMISTRY, GENETICS
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Original article | Digestive Diseases

**PREVENTIVE ZINC SUPPLEMENTATION EFFECT
ON REDOX STATUS IN RAT MODEL OF MAFLD**

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Background. Oxidative stress plays an important role in the pathogenesis of metabolic-associated fatty liver disease (MAFLD). Antioxidant trace elements as cofactors of antioxidant enzymes and metalloproteins are involved in this process. Zinc being an important antioxidant may have a positive effect on the treatment of liver pathology. The study aimed to assess the effect of preventive zinc supplementation on MAFLD in rats.

Materials and Methods. A total of 26 three-month-old female Wistar rats were used in the present study. The activity of the antioxidant enzymes superoxide dismutase and catalase, some redox status markers, such as ceruloplasmin, oxidized tryptophan, dithyrosines, total thiols, carbonyls, TBARS, and uric acid were evaluated. Oxidative stress biomarkers were studied spectrophotometrically.

Results. MAFLD was accompanied by hyperuricemia and a decrease in serum dityrosines. The addition of Zn to the diet prevented the development of steatosis, decreased the level of oxidized tryptophan in the liver, and paradoxically caused hyperuricemia in the MAFLD model used. Zn supplementation had a positive effect on the prevention of MAFLD, had a little effect on redox status of animals but caused paradoxical hyperuricemia. Future studies are needed to establish the mechanisms of the Zn effect at the cellular level.

Keywords: MAFLD; redox status; liver steatosis; zinc; uric acid

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Научная статья | Болезни органов пищеварения

ВЛИЯНИЕ ПРОФИЛАКТИЧЕСКОГО ПРИМЕНЕНИЯ ЦИНКА НА ОКИСЛИТЕЛЬНО-ВОССТАНОВИТЕЛЬНЫЙ СТАТУС В МОДЕЛИ MAFLD

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Обоснование. Окислительный стресс играет важную роль в патогенезе метаболически-ассоциированной жировой болезни печени (MAFLD). В этом процессе участвуют антиоксидантные микроэлементы в качестве кофакторов и металлопротеинов. Цинк, являясь важным антиоксидантом, может оказывать положительное действие при патологии печени.

Цель. Оценить влияние профилактического применения цинка на некоторые окислительно-восстановительные параметры и морфологию печени на модели MAFLD у крыс.

Материалы и методы. В исследовании использовали 26 трехмесячных самок крыс линии Вистар. Оценивали активность супероксиддисмутазы и каталазы, содержание церулоплазмينا, окисленного триптофана, дитиозинон, общих тиоловых и карбонильных соединений, ТБК-РС и мочевую кислоту на спектрофотометре. Статистическая обработка данных осуществлялась с помощью RStudio для MacOS (версия 1.3.1056).

Результаты. MAFLD сопровождалась гиперурикемией и снижением уровня дитиозинон в сыворотке крови. Добавление Zn в рацион предотвращало развитие стеатоза, снижало уровень окисленного триптофана в печени, но вызывало гиперурикемию в используемой модели MAFLD. Применение цинка оказало положительный эффект при профилактике MAFLD, мало влияло на окислительно-восстановительный статус животных, но вызвало парадоксальную гиперурикемию. Поэтому, необходимы дальнейшие исследования для установления механизмов действия цинка на клеточном уровне.

Ключевые слова: MAFLD; окислительно-восстановительный статус; стеатоз печени; цинк; мочевая кислота

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Introduction

Metabolic associated fatty liver disease (MAFLD) also termed nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the world with a global prevalence estimated at 24% [1]. MAFLD is defined as a hepatic manifestation of a complex pathological condition in which more than 5% of hepatocytes contain fat droplets. It is associated with multimetabolic disorders [6], hypertension, insulin resistance, diabetes, dyslipidemia, and obesity [11, 19, 20]. Based on this idea and the recent advantages in disease pathogenesis a recent consensus from an international panel proposed a new definition for NAFLD: MAFLD [30].

Trace elements are necessary to maintain the redox status, for intracellular signaling, implementation of enzyme activity, etc. [25, 27] and an imbalance in their metabolism may play a significant role in MAFLD and related diseases [3]. Significant negative association of Zn with the severity of MAFLD has been shown [2]. Zn plays an important role in stabilizing insulin hexamers, accumulation of pancreatic hormones and signaling pathways involved in insulin action [4]; it is an efficient antioxidant [10]. Insulin resistance has traditionally been defined as one of the main pathophysiological factors of MAFLD and previous studies have demonstrated that oxidative stress and endoplasmic reticulum stress were associated with disease pathogenesis [15].

Objective

Taking into account all of this evidence, the preventive use of Zn in MAFLD may reduce the severity of the metabolic changes that underlie the excessive accumulation of lipids in hepatocytes. Therefore, the aim of this study was to assess the effect of preventive Zn supplementation on the diet-induced model of MAFLD in rats.

Material and methods

Experimental design

In the present study, a total of 26 three-month-old female Wistar rats were used. The study protocol was approved by the institutional Local Ethics Com-

mittee (protocol № 1/14.01.2017) and carried out in accordance with the ethical standards laid down in the Declaration of Helsinki of 1964 and its subsequent amendments. The animals were acclimatized to laboratory conditions for 2 weeks prior to the experiment. The temperature in the animal room was $23 \pm 1^\circ\text{C}$. The light and dark cycles in the animal room were 12 each (8.00–20.00). The total duration of the dietary intervention was 4 weeks. All rats were fed a standard laboratory chow (270 kcal/100 g, 20% kcal protein, 7% kcal carbohydrates, 10% kcal fat, 15.01% of crude fiber) “Orenburg food mixture factory”, Orenburg, Russia). Laboratory chow was composed of wheat (at least 10%), oat (at least 10%), bran (no more than 20%), sunflower oil cake (at least 10%), husk, calcium phosphate, calcium carbonate, sodium chloride, and vitamin mix (vitamins A, D₃, B1, B2, B3, B4, B5, B6, B12, folic acid, and biotin), amino acids methionine, cysteine, threonine. The Zn level in the diet was 30 µg/g.

The animals were divided into 4 groups:

- 1) Control (C) (n = 6) were kept on standard chow (10% fat calories, 270 kcal/100 g, ‘Orenburg Food Mixture Factory’, Orenburg, Russia) and pure drinking water (total mineralization of less than 250 mg/L).
- 2) Animals from the second group (MAFLD) (n=6) developing MAFLD were maintained on a lard-based high-fat high-carbohydrate diet (60% calories from fat (429 kcal/100 g) and 10% sucrose solution instead of drinking water (40 kcal/100 ml)).
- 3) Zn-supplemented group of animals supplemented with Zn (ZnS) (n = 7) obtained 227 mg / L of zinc daily as Zn sulfate ZnSO₄ dissolved in drinking water
- 4) Animals from group number four (MAFLD-ZnS) (n=7) were maintained on a lard-based high-fat high-carbohydrate diet (60% calories from fat (429 kcal/100 g) and 10% sucrose solution and 227 mg/L zinc as Zn sulfate ZnSO₄ instead of drinking water (40 kcal/100 ml)).

Histological Assessment of the Liver

At the end of the study, liver samples were fixed in neutral buffered formalin and embedded in paraffin blocks. Obtained samples were sliced (5 µm) and stained with hematoxylin and eosin. Histology of liver tissue was examined using an Olympus CX31 UTV1X2 & UCMAD3 (Japan) microscope equipped with a digital camera. ImageJ software (NIH, Bethesda, MD, USA) software was used for the analysis of hepatocyte nucleus areas. For the purpose of analysis, the nuclear circumferences were measured (100 nuclei per image – in the periportal and centrilobular zones of each liver sample). Nucleus area was calculated from nuclear circumference by the ImageJ software and expressed as µm².

Oxidative stress biomarkers

Serum and liver samples were used for the assessment of oxidative stress biomarkers. The analysis of SOD activity was based on the registration of adrenochrome (a product of adrenaline autoxidation) at 347 nm [18]; catalase activity was determined at 410 nm by the reaction of H₂O₂ with ammonium molybdate [12]; the ceruloplasmin content was assayed using the Ravin method with p-phenylenediamine in sodium hydroxide as substrate and expressed as mg/g protein [26]. TBARS content was assessed in the reaction with thiobarbituric acid [21]; TSH content was determined by using thiol-specific reagent dithionitrobenzoic acid (DTNB) at 412 nm [17]; carbonyls content was assayed in reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) with the formation of protein-bound 2,4-dinitrophenylhydrazones at 370 nm [14]; dityrosine and oxidized tryptophan concentrations were assessed in presence of FeSO₄ and EDTA by registration of blue dityrosine fluorescence formed due to oxidation of tyrosine residues at the excitation wavelength 325 nm, emission wavelength 415 nm and decreased tryptophan fluorescence at the excitation wavelength 295 nm, emission wavelength 340 nm [32].

Liver homogenization (1:5; w/v) was performed in an ice-cold 1/15 M phosphate buffer (pH = 7.4) with subsequent centrifugation (4000 × g, 15 min, 4°C). Serum and supernatant levels of TBARS, TSH, and carbonyls were analyzed spectrophotometrically at PD-303UV spectrophotometer (Apel, Japan). SOD and catalase activity in the supernatant were assessed spectrophotometrically at SF-56 UV/Vis spectrophotometer (OKB Spectr, Saint Petersburg, Russia). Serum and supernatant levels of dityrosine and oxidized tryptophan were assessed by means of fluorometry analysis at Fuorat ABLF 2 fluorimeter (Lumex, Saint Petersburg, Russia). Serum uric acid content was measured spectrophotometrically using the respective Vector-Best kit (Vector-Best, Novosibirsk, Russia).

Statistical analysis

Statistical analysis was performed by RStudio for MacOS (version 1.3.1056), an open-source of software, programmed in the R programming language. Data were expressed as median and 25 and 75 percentile boundaries. Group-by-group comparison of data was evaluated using the Kruskal-Wallis test with the post hoc Dunn test for multiple comparisons. All differences were considered significant at p < 0.05.

Results

Histological Assessment of the Liver

As it is seen from Fig. 1, mean nucleus area in centrilobular and periportal zones was lower than that in the control group in rats with MAFLD.

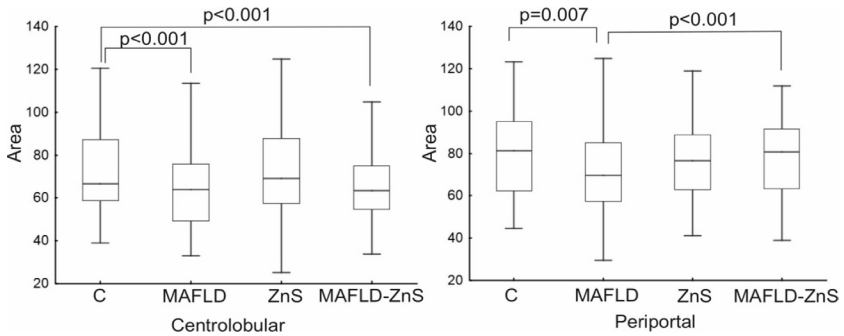


Fig. 1. Analysis of the hepatocyte nucleus areas (in μm^2). Data presented as median (25-75) (box) and non-outlier ranges.

Zn treatment resulted in a significant increase in periportal nucleus area in MAFLD-Zn group accompanied by a decrease in steatosis in this zone (Fig. 2).

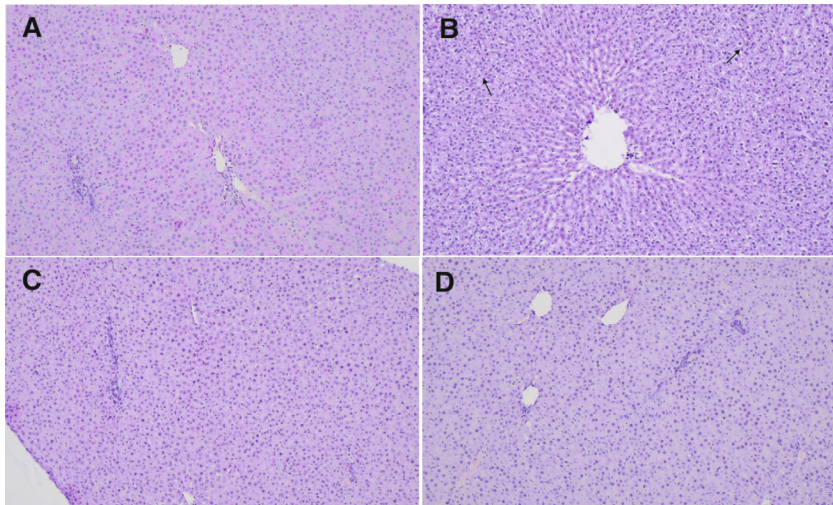


Fig. 2. Liver histology (hematoxylin and eosin stain, $\times 100$). a – control animals; b – MAFLD animals; c – ZnS animals; d – MAFLD-ZnS animals. Zones of steatosis are shown by arrows

Oxidative stress markers of the liver

The data obtained indicate that Zn supplementation significantly affected the parameters of oxidative stress in the liver (Table 1). In particular, liver oxidized

tryptophan levels in ZnS and MAFLD-ZnS groups were significantly lower than that in control group by 33 ($p=0.005$) and 37% ($p=0.018$), respectively. Liver total thiols (TSH) content was reduced in ZnS group being 16% ($p=0.046$) less in comparison with the control values.

Table 1.

Liver oxidative stress parameters of experimental animals

Parameter	C	MAFLD	ZnS	MAFLD-ZnS
Liver SOD, U/min*mg protein	314.9 (194.3-315.0)	159.1 (118.3-173.4)	222.8 (152.5-320.6)	192.9 (118.9-217.5)
Liver ceruloplasmin, mg/g protein	4.6 (4.6-5.7)	3.3 (3.1-3.7)	5.5 (4.7-5.7) ^b	3.7 (3.7-4.4)
Liver catalase activity, $\mu\text{mol/l}^*\text{g protein}$	296.4 (257.5-308.9)	324.2 (310.1-326.8)	280.0 (265.7-320.3)	260.8 (224.3-390.1)
Liver oxidized tryptophan, U/g protein	1.4 (1.2-1.5)	1.1 (0.9-1.4)	0.9 (0.8-1.0) ^a	0.9 (0.8-1.0) ^a
Liver dityrosines, U/g protein	1.0 (0.9-1.1)	1.1 (0.8-1.6)	0.8 (0.5-1.1)	1.1 (0.9-1.6)
Liver TBARS, nmol/mg protein	1.2 (1.1-1.3)	1.2 (1.2-1.5)	1.0 (0.8-1.3)	1.4 (1.2-1.4)
Liver TSH, mmol/mg protein	11.3 (10.7-11.5)	10.0 (9.8-10.6)	9.5 (8.8-10.2) ^a	9.7 (9.2-11.3)
Liver protein carbonyls, $\mu\text{mol/l}$	23.3 (16.5-26.4)	22.9 (16.8-23.3)	16.574 (14.8-22.0)	17.5 (14.1-20.8)
Data presented as median (25-75); a – significant difference compared to control animals ($p < 0.05$); b – significant difference compared to MAFLD animals ($p < 0.05$); c – significant difference compared to ZnS animals ($p < 0.05$)				

Oxidative stress markers of the serum

As it is seen from Table 2, Zn supplementation or MAFLD did not affect significantly serum TBARS, TSH and carbonyls content that indicate an absence of oxidative protein modification in rats' serum. However, the serum dityrosines content in overfed rats (MAFLD) with both absence and presence Zn treatment was significantly lower than the control values by 34% ($p=0.013$) and 38% ($p=0.024$), respectively.

Despite a 23 ($p=0.121$) and 35% ($p=0.648$) decrease in serum ceruloplasmin concentration in the MAFLD and MAFLD-ZnS groups, the observed changes were not significant. Uric acid content was 39% higher in rats with MAFLD than that in the healthy animals ($p=0.063$). Surprisingly, Zn supplementation resulted in 2 ($p=0.070$) and a more than 2-fold increase ($p=0.005$)

in serum uric acid concentration in the ZnS and MAFLD-ZnS groups, respectively.

Table 2.

Serum oxidative stress parameters of experimental animals

Parameter	C	MAFLD	ZnS	MA-FLD-ZnS
Serum ceruloplasmin, mg/g protein	2.5 (2.2-2.8)	1.9 (1.8-2.1)	2.3 (2.2-2.4)	1.6 (1.5-2.9)
Serum oxidized tryptophan, U/g protein	0.025 (0.023-0.029)	0.022 (0.021-0.023)	0.024 (0.020-0.027)	0.023 (0.020-0.024)
Serum dityrosine, U/g protein	0.032 (0.026-0.039)	0.021 (0.018-0.023) ^a	0.030 (0.026-0.034) ^b	0.020 (0.019-0.024) ^a
Serum TBARS, nmol/mg protein	0.24 (0.18-0.29)	0.41 (0.20-0.80)	0.26 (0.23-0.52)	0.42 (0.19-0.52)
Serum TSH, mmol/mg protein	0.87 (0.84-1.20)	0.92 (0.70-1.33)	1.12 (0.86-1.23)	1.09 (0.98-1.31)
Serum protein carbonyls, μ mol/l	59.8 (53.3-75.9)	55.9 (50.5-59.1)	53.9 (41.4-68.4)	61.5 (37.6-82.5)
Uric acid, μ mol/l	155.2 (132.2-166.7)	255.8 (224.1-678.2)	310.3 (195.4-321.8)	327.6 (316.1-431.0) ^a
Data presented as median (25-75); a – significant difference compared to control animals ($p < 0.05$); b – significant difference compared to MAFLD animals ($p < 0.05$); c – significant difference compared to ZnS animals ($p < 0.05$)				

Discussion

Changes in levels of certain trace elements such as copper, zinc, and iron play a crucial role in the occurrence of metabolic disorders. Disturbance of trace elements homeostasis is recognized as one of the mechanisms responsible for MAFLD development [29]. Since the liver is the main organ of lipid metabolism and metal biotransformation, hepatic dysfunction can be closely related to MAFLD through oxidative stress (Haber-Weiss and Fenton reactions) [8, 9]. In the present study we did not reveal any changes in liver redox status in animals with MAFLD. The existing data on redox enzymes activity in liver steatosis are very contradictory. A detailed clinical study by Videla et al. (2004) demonstrated more than a 4-fold increase in liver protein carbonyls in patients with steatosis compared to healthy controls [33]. The authors showed a decrease in SOD and catalase activity in patients with steatosis and steatohepatitis [33]. The study by Yesilova et al. (2005) revealed a negative association between Cu/Zn-SOD levels and BMI, glucose, insulin, and HO-

MA-IR in MAFLD [34]. In a previous study, the same results with unchanged TBARS, TSH, and carbonyls were shown [7]. The possible reason for the results obtained could be an initial stage of the disease when there were no significant changes in liver metabolism.

Zn treatment reduced liver oxidized tryptophan both in the healthy animals and rats with MAFLD, and TSH levels in healthy animals. These results are in line with those of previous studies indicating an antioxidant effect of Zn [22]. It was shown that ionic Zn binds to thiol groups of proteins and prevents their oxidation [24]. However, the mechanism of the revealed decrease in oxidized tryptophan requires a more detailed study.

Although serum uric acid levels showed a tendency to increase in healthy animals treated with Zn and rats with MAFLD, data on the role of uric acid in MAFLD, metabolic syndrome, and other metabolic disorders are controversial. Our finding corresponds to the existing data showing fructose-induced hyperuricemia in MAFLD [13]. In a study by Oral et al., BMI, homeostatic assessment model (HOMA-IR), and uric acid values were significantly higher in the NAFLD group than in the control group. A positive correlation was found between stage NAFLD and uric acid concentration [23]. At the same time, many studies have indicated that uric acid is a strong natural scavenger of various free radicals [5]. Uric acid is an end product of purine metabolism, and its homeostasis depends on production and excretion. Excretion is mainly regulated by glomerular filtration and reabsorption [16]. The driving force behind the reabsorption by the uric acid transporter URAT1 is the exchange of uric acid with chloride, sulfate, and other anions [28]. In our opinion, zinc sulfate used in the study could induce an increased excretion of sulfate anions in urine, competing with uric acid, and as a consequence, provide an increased level of its reabsorption resulting in the observed rise. Umeki et al. showed that oral zinc reverses elevated serum uric acid concentration to normal levels in patients with Wilson's disease, possibly by increasing synthesis in the liver [31]. However, data obtained from the NHANES (2001-2014) reveal an inverse association between zinc intake and hyperuricemia among adults in the United State [35]. In our opinion, more detailed studies are needed to understand the Zn effect on purine metabolism.

In general, the results of the study showed that Zn supplementation prevented liver steatosis developed in MAFLD, reduced liver oxidized tryptophan in the healthy animals and rats with MAFLD. In addition, Zn treatment in healthy animals and MAFLD-ZnS treated animals resulted in unexplained hyperuricemia

Conclusions

In conclusion, Zn supplementation improved liver morphology in rats with MAFLD, but causes paradoxical hyperuricemia. At the same time, no significant changes in liver redox status and an only slight effect on serum oxidative stress markers were observed in the present model of MAFLD. Future studies are needed to establish the mechanisms of the Zn effect at the cellular level.

Author contributions. The authors contributed equally to the research.

Conflict of interest statement. The authors declare that they have no conflict of interest.

Availability of data and material. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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