

Lipoic Acid Decreases 3-Nitrotyrosine and Cytokine Levels In A Rat Sepsis Model

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ABSTRACT

It was aimed to investigate the effects of lipoic acid (LA) on 3-nitrotyrosine (3-NT), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), magnesium and trace elements levels in serum of rats with sepsis.

Control group ($n=10$) received a single dose of saline, lipopolysaccharide (LPS) group ($n=10$) was injected a single dose of 20 mg/kg LPS, and LA+LPS ($n=10$) group was injected a single dose of 20 mg/kg LPS at the end of third day of 10 mg/kg/day LA injection. The serum 3-NT levels were determined by a high performance liquid chromatography. The serum levels of IL-1 β and TNF- α were measured by enzyme linked immunosorbent assay. Determination of magnesium and trace element levels were performed by inductively coupled plasma mass spectrometer.

3-NT, IL-1 β and TNF- α levels in control and LA+LPS groups were not determined. However, observable levels of these parameters were detected in the LPS group. It was found that manganese levels were positively correlated with IL-1 β and TNF- α in LPS group. LA increased the manganese levels, while it decreased magnesium and iron levels in LA+LPS group.

Our results show that LA decreases serum levels of 3-NT, IL-1 β and TNF- α in rats with sepsis, and it is also effective on trace element levels. Decreased levels of manganese and its association with IL-1 β and TNF- α in the LPS group suggest that manganese may be associated with immune response.

Key Words: Sepsis, lipoic acid, 3-nitrotyrosine, tumor necrosis factor-alpha, interleukin-1 β

Introduction

Sepsis is a complex pathophysiological condition resulting from a systemic inflammatory response to infection and is the leading cause of death in critically ill patients (1). In this condition, phagocytic cells firstly react with the lipopolysaccharide (LPS) or endotoxin (2). In response to LPS, macrophages excrete proinflammatory cytokines including tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). Tumor necrosis factor-alpha seems to be important for diverse effects, which stimulates inducible nitric oxide synthase (iNOS) excretion (3). Nitric oxide can interact with superoxide (O₂⁻) to compose the peroxynitrite (ONOO⁻) leading to septic shock with high mortality after a number of mechanisms. The measurement of 3-nitrotyrosine (3-NT) can mirror the stage of ONOO⁻ induced tissue damage (4). The relationship between 3-NT and nitrosative tissue damage in sepsis has also been documented in recent studies using experimental models of endotoxemia (4, 5).

Trace elements are needed for the efficiency of a number of immune cells and acute phase proteins

that directly interact and contribute to protection processes during infection (6). Several trace elements are toxic, while others are characterized with anti-inflammatory abilities (6, 7). According to recent investigations, there have been a few elements involved in inflammatory diseases such as zinc and selenium (4, 6, 7). It is therefore acceptable to offer that these elements might also impact inflammatory responses (6, 7).

Lipoic acid (LA) has been demonstrated to be advantageous in inhibition of inflammation intervening pathological circumstances (8). Following investigations have demonstrated that LA does not only participate in the recycling of vitamin E and glutathione but also scavenges chelate transition metals and hydroxyl radicals (9). On the other hand, another attractive property of LA eliminates reactive oxygen species in both lipophilic and hydrophilic surroundings because it is both water and fat soluble (10). LA effectively attenuates LPS-induced acute inflammatory response in lung tissues in LPS-induced sepsis and a cecal ligation (11, 12).

Although it is completely accepted that essential trace elements are necessary for performance,

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activation and differentiation of countless missions of immune cells, the specific roles of these trace elements in these processes remain largely ambiguous (7). Moreover, the relationship between trace elements and inflammatory marker levels has not been determined in sepsis previously. Above all, the effects of LA on 3-NT, magnesium and trace elements levels have not been studied in sepsis yet. Considering these aspects, we aim to investigate the effects of LA on magnesium, trace elements, 3-NT, IL-1 β and TNF- α levels in rats with LPS induced sepsis.

Materials and Methods

Animals and Experimental Design: This investigation was funded by the Erciyes University Scientific Research Projects Unit (TSA-08-590). The investigations were conducted on 30 adult male wistar rats weighing 260–310 g. A maximum of six rats can be hosted in each cage to ensure standard conditions. Thus, each group (n=10) was divided into two cages; as a result, 5 rats were placed in each cage, with an ambient temperature of 20 \pm 2 $^{\circ}$ C, humidity of 55 \pm 5% and a 12/12 h dark-light cycle (dark on at 19:00 p.m.). The rats were fed with a standard food and water ad libitum. Before the start of the experiment, all rats were given a seven-day adaptation period. The experimental procedures followed the European Community guidelines as accepted regulations for using the animals. The animal protocols used in this study were examined and confirmed by Erciyes University Experimental Animals Ethics Committee (5-08/25-09.04.2008). The experimental procedures were performed according to Local Ethics Committee Guidelines of Animal Experiments of Erciyes University (Law no.: 2911/2007). The animals were randomly separated into three groups.

In some groups, it was reported that iNOS induction reached maximum level at 6th hour after the LPS injection (13, 14). Since animals in Group II and Group III had stress conditions during injection, saline injection was applied to create a similar condition in Group I. In view of the literature, blood samples of Group II and Group III were taken at 6th hour after LPS injection. Similarly, blood samples of Group I were taken at 6th hour after saline injection. The required procedures were applied to groups as follows:

Group I: Control group animals (n=10) were injected with single dose of 20 mg/kg saline intraperitoneally (IP). Later, blood specimens were

taken under anesthesia with xylazine/ketamine at 6th hour after saline injection.

Group II: LPS group animals (n=10) were injected with LPS intraperitoneally single dose of 20 mg/kg (15). Later, blood specimens were taken under anesthesia with xylazine/ketamine at 6th hour after LPS injection (16).

Group III: LA+LPS group animals were injected with LA intraperitoneally for three days at a dose of 10 mg/kg/day. At the end of the third day of LA injection, these animals were injected with LPS a single dose of 20 mg / kg intraperitoneally. Later, blood specimens were taken under anesthesia with xylazine/ketamine at 6th hour after LPS injection (13, 16).

Measurement of 3-Nitrotyrosine: The serum nitrotyrosine levels were determined by High Performance Liquid Chromatography (HPLC) technique implemented by Çimen et al. (13) as follows: 0.3 ml of serum specimen was taken for this operation to precipitate protein, and an addition of 0.3 ml of 10% TCA on serum specimen. After centrifuging for 10 min at 3000 rpm, protein was hydrolyzed at 100 $^{\circ}$ C for 18–24 h in 6 N HCl. Samples were taken into specific hydrolysis tubes after sonication. The samples were determined on a diode array detector (Hewlett Packard, Germany). The column had 5 μ m pores ODS-2 C18 reverse phase (Alltech, USA) (16).

Determination of IL-1 β and TNF- α : The serum levels of IL-1 β and TNF- α were determined by using enzyme linked immunosorbent assay test. These kits (IL-1 β : Invitrogen Rt-Catalog Number: KRC0011 and TNF- α : Invitrogen Rt-Catalog Number: KRC3011) use ELISA based monoclonal antibody specific for IL-1 β and TNF- α . The measurements were performed briefly as follows; controls and blood specimens were pipetted into wells and incubated. Biotinylated antibodies were added into wells and incubated. Streptavidin-Peroxidase was added after elimination of excessive antibodies. A substrate, which acted upon the bound enzyme to produce color after elimination of unbound enzyme, was added. The color intensity of product was directly proportional to the IL-1 β and TNF- α levels.

Measurement of Magnesium and Trace Element Levels: Determination of Mg and trace metal ions in serum samples (Cu, Fe and Mn) was carried out by means of inductively coupled plasma mass spectrometer (Agilent Technologies, Tokyo, Japan). The method was performed using the action defined by Isabel De Blas Bravo et al.

Table 1. Serum concentrations of magnesium and trace elements

Parameters	Groups			Comparisons		
	LPS (n=10) Median (Min- Max)	LA+LPS (n=10) Median (Min- Max)	Controls (n=10) Median (Min- Max)	LPS / Control	LA+LPS / Control	LPS / LA+LPS
Mg (ppm)	29.9(21.1-50.5)	26.1(17.4-47.7)	13.9(6.36-33.4)	p=0.009	p=0.041	p=0.143
Cu (ppm)	0.093(0.024-0.095)	0.35(0.34-0.35)	0.08(0.001- 0.172)	p=0.909	p=0.127	p=0.180
Fe (ppm)	9.52(5.92-17.9)	7.07(3.15-12.9)	2.41(1.57-2.99)	p<0.001	p<0.001	p=0.199
Mn (ppm)	0.007(0.001-0.050)	0.06(0.05-0.47)	0.04(0.03-0.05)	p=0.023	p=0.048	p=0.011

Values are median (min-max) for all parameters

(17). The detection limits of the method were found to be 0.14, 0.18, 0.04, and 0.07 ng/mL of assay solution for Mg, Cu, Fe and Mn, respectively.

Statistical Analysis: SPSS software version 15.0 and SigmaStat 3.5 (IBM Corp., New York, USA) statistics programs were used for statistical analyses. Kolmogorov-Smirnov test was used for normality of data distribution. Study groups, without a normal distribution, were compared to each other with the Kruskal–Wallis test followed by post hoc test. However, the levels of 3-NT, IL-1 β and TNF- α were not detected in the control and LA+LPS groups. Therefore, study groups could not be compared statistically in terms of 3-NT, IL-1 β and TNF- α levels. Pearson or Spearman correlation test was conducted according to the distribution of the data to investigate the relationship between parameters. Continuous variables were expressed as median (min-max); and categorical variables were expressed as number. Statistical significance was set at 0.05.

Results

Plasma concentrations of 3-NT, IL-1 β and TNF- α were quantified to assess systemic inflammatory responses in LPS and LA+LPS groups. We did not detect serum levels of 3-NT, IL-1 β and TNF- α in control group. The same as the control group, serum levels of 3-NT, IL-1 β and TNF- α were not detected in LA+LPS group. On the other hand, 3-NT level was measured as 2.69(2.60-2.92 μ mol/L), IL-1 β level was measured as 48.80 (10.30-245.20 pg/ml) and TNF- α level was measured as 29.80 (11.20-114.20 pg/ml) in LPS group [median(min-max)]. In terms of serum levels of Cu, we found that there were not any

significant differences among controls [0.08(0.001-0.172)], LPS [0.093(0.024-0.095)] and LA+LPS [0.35(0.34-0.35)] groups as median (min-max). The serum levels of Mg and Fe were significantly higher in LPS [29.9(21.1-50.5); 9.52(5.92-17.9), respectively] and LA+LPS [26.1(17.4-47.7); 7.07(3.15-12.9), respectively] than those of the controls [13.9(6.36-33.4); 2.41(1.57-2.99), respectively] as median (min-max). There were not any significant differences between LPS and LA+LPS groups. The serum levels of Mn were significantly lower in LPS [0.007(0.001-0.050)] than those of the controls [0.04(0.03-0.05)] and LA+LPS [0.06(0.05-0.47)] as median (min-max). On the other hand, the serum levels of Mn were significantly higher in LA+LPS than those of the controls (Table 1).

When correlation analyses were performed to investigate the association of Cu, Fe, Mg and Mn with 3-NT, IL-1 β and TNF- α in LPS, there were not any statistically significant correlations between Cu, Fe and Mg with 3-NT, IL-1 β and TNF- α and in controls, LPS and LA+LPS. On the other hand, Mn levels significantly positively correlated with IL-1 β and TNF- α in LPS group ($r=0.784$, $p=0.012$; $r=0.883$, $p=0.001$, respectively; Figure 1, Figure 2). Moreover, IL-1 β levels were significantly correlated with TNF- α in LPS group ($r=0.741$, $p=0.022$; Figure 3).

Discussion

This study demonstrated the protective effects of LA in sepsis by non-detectable serum levels of 3-NT, IL-1 β and TNF- α in LA+LPS group similar to the control group. However, 3-NT, IL-1 β and TNF- α serum levels were detected in LPS group. On the other hand, we also found that levels of Fe, Mg in LPS increased, while levels of Mn

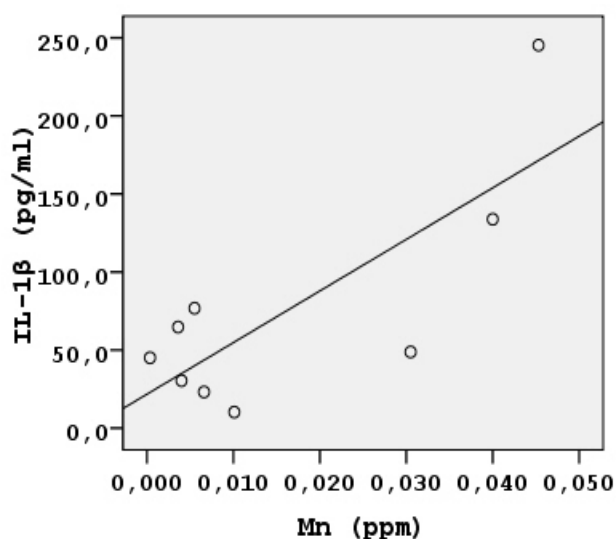


Fig. 1. Correlations of manganese (Mn) with interleukin-1 β (IL-1 β) in lipopolysaccharide group ($r=0.784$, $p=0.012$).

decreased and it had relationships with IL-1 β and TNF- α in LPS group.

Previous studies showed that α -LA inhibited the symptom of oxidative stress and inflammatory mediators, such as TNF- α and iNOS (18). Some other studies demonstrated that LA administration decreased IL-1, IL-6, IL-1 β and TNF- α levels (15-17). We measured 3-NT, IL-1 β and TNF- α at 6th hour after LPS injection in all groups. We demonstrated that IL-1 β and TNF- α levels were measurable in the LPS group compared to controls and LA+LPS groups. Moreover, IL-1 β levels showed statistically significant correlations with TNF- α in LPS group. Previously, researchers proved that reduced energy stores began to increase after 12-24 hours to reach the control values after LPS injection (17). Some studies also reported that iNOS induction reached maximum level at 6th hour after the LPS injection and fell sharply after 12 hours and returned to almost undetectable levels after 24 hours (13, 17). The evaluation of ONOO⁻ induced tissue damage could be monitored by measuring 3-NT with different approaches due to very short life expectancy of ONOO⁻ (18, 19, 20). 3-NT was measured with immunostaining experiments, but no labeling of iNOS and 3-NT in histologically normal liver tissue were detected (21). In this aspect, our findings are consistent with previous studies (19, 21). The ONOO⁻ failure, which may occur as a result of inhibition of iNOS by LA, may prevent the formation of 3-NT as found in our study.

Previous researchers found that LA effectively diminished acute inflammation induced by LPS;

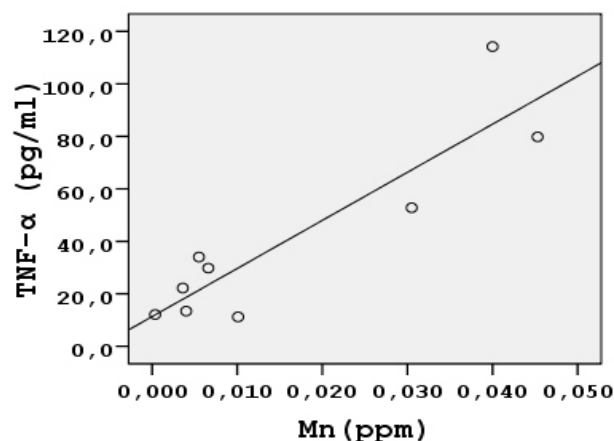


Fig. 2. Correlations of manganese (Mn) with tumor necrosis factor-alpha (TNF- α) in lipopolysaccharide group ($r=0.883$, $p=0.001$)

and it was suggested that LA could be a novel therapeutic agent in protection of inflammatory vascular diseases induced with endotoxemia (12). In our study, lipoic acid was not used for therapeutic purposes; however, we used the LA in rats for protective purposes. Although 3-NT levels in serum were not detected in control and LA+LPS groups, 3-NT levels were measured in the LPS group. Considering these aspect, undetectable levels of 3-NT, IL-1 β and TNF- α in LA+LPS and controls are consistent with the previous reports and it may demonstrate the protective effect of LA in sepsis (18).

Perhaps one of the most important finding of our study is the demonstration of the trace elements levels and their changes by the effect of LA and also their relationship with IL-1 β and TNF- α in study groups. There are previous researches in which the relationship between trace elements and sepsis was investigated (22, 23). Some studies reported a decrease in trace element levels with sepsis; while others found an increase in Zn and Cu with sepsis (23, 24). Nevertheless, the impacts of LA on the trace element levels or its metabolism have not been studied in sepsis. Our study demonstrated that sepsis has increased the levels of Fe and Mg, while Mn *concentration was* decreased in LPS group. Moreover, our results have showed that Fe and Mg levels in serum with LA+LPS group have not returned to control levels with treatment of LA 10 mg/kg/day.

The increased levels of Fe and Mg in LPS and LA+LPS groups might be associated with the simultaneous decrease of these elements in the lung and other tissues.

The existence of E.coli-mediated lipid peroxidation in peritoneum has been detected; and

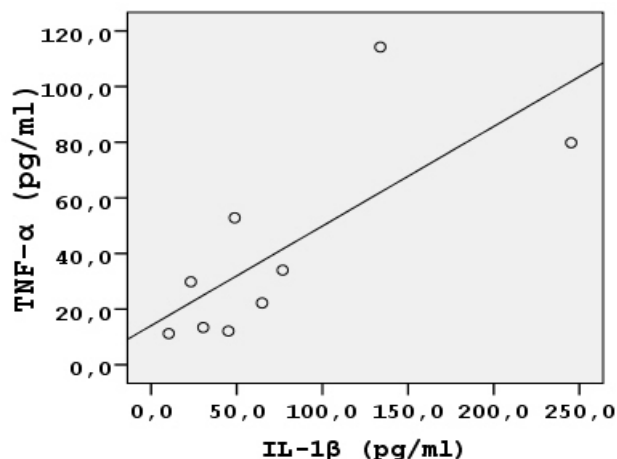


Fig. 3. Correlations of interleukin-1 β (IL-1 β) with tumor necrosis factor-alpha (TNF- α) in lipopolysaccharide group ($r=0.741$, $p=0.022$)

this is accompanied by an increase in Cu, Fe and Zn levels (25). Another study has found that induced sepsis by cecal ligation and sepsis have significantly reduced levels of Zn and Cu in all tissues (22). Iron is a necessary nutrition for microorganism and the environment of the gastrointestinal tract of mammalian. Alterations in distribution and availability of Fe have significant influences on the response of the defense system (26). Mg is a good modulator of the immune system; and significantly enhanced levels of it in infected tissues have been demonstrated (27).

Our findings also show that Mn levels statistically significantly positively correlate with IL-1 β and TNF- α in LPS group since manganese is a beneficial trace element needed for numerous cellular processes such as insulin release and formation of many important enzymes and is also a critical cofactor of many enzymes in carbohydrate metabolism (28). A previous research indicated that vertebrates accumulate Zn, Mn and Fe both extracellularly and intracellularly to defend themselves against infections (29). Our findings are consistent with these evidences although, probably, Mn and Fe levels were triggered by different mechanisms since only Mn levels decreased with sepsis in our study. The findings of the present study also indicate that LA administration increases the Mn levels in sepsis while it decreases the levels of Mg and Fe in sepsis.

Nevertheless, the present study has several restrictions, including the fact that the study groups could not be compared statistically in terms of 3-NT, IL-1 β and TNF- α levels. As a result of this, parameter levels were below the measurement limit. Another limitation is our inability to analyze more cytokine and oxidative

stress markers in the study due to budgetary constraints.

All in all, these results may be interpreted as having protective effect of LA on the levels of 3-NT formation resulting from the expression of iNOS and cytokine induction. On the other hand, it may suggest an increase in Mg and Fe levels which are probably involved in the immunological host response and decreased levels of Mn and its relationships with IL-1 β and TNF- α in LPS group may show that Mn is related with the immune response. So, we suggest that LA supplementation may be beneficial for prevention of inflammation mediated pathological conditions. However, the sepsis-induced damages have not sufficiently been emphasized to control levels in terms of Fe and Mg levels with treatment given LA dose (10 mg/kg/day). Therefore, future investigations may be needed to prevent the tissue damage; and the given LA dose must be adjusted.

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References

1. Bhan C, Dipankar P, Chakraborty P, Sarangi PP. Role of cellular events in the pathophysiology of sepsis. *Inflamm Res* 2016;65:853-868.
2. Zhang C, Walker LM, Mayeux PR. Role of nitric oxide in lipopolysaccharide-induced oxidant stress in the rat kidney. *Biochem Pharmacol* 2000;59:203-9.
3. Eigler A, Moeller J, Endres S. Exogenous and endogenous nitric oxide attenuates tumor necrosis factor synthesis in the murine macrophage cell line RAW264.7. *J Immunol* 1995;154:4048-54.
4. Hensley K, Williamson KS, Floyd RA. Measurement of 3-nitrotyrosine and 5-nitro-gamma-tocopherol by high-performance liquid chromatography with electrochemical detection. *Free Radic Biol Med* 2000;28:520-8.
5. Shi Q, Cao J, Fang L, et al. Geniposide suppresses LPS-induced nitric oxide, PGE2 and inflammatory cytokine by downregulating NF- κ B, MAPK and AP-1 signaling pathways in macrophages. *Int Immunopharmacol* 2014;20:298-306.
6. Mal P, Dutta K, Bandyopadhyay D, Basu A, Khan R, Bishayi B. Azithromycin in combination with riboflavin decreases the

- severity of *Staphylococcus aureus* infection induced septic arthritis by modulating the production of free radicals and endogenous cytokines. *Inflamm Res* 2013;62:259-73.
7. Failla ML. Trace elements and host defense: recent advances and continuing challenges. *J Nutr* 2003; 133:1443-7.
 8. Tibullo D, Li Volti G, Giallongo C, et al. Biochemical and clinical relevance of alpha lipoic acid: antioxidant and anti-inflammatory activity, molecular pathways and therapeutic potential. *Inflamm Res* 2017; 66:947-959.
 9. Kagan VE, Kuzmenko AI, Shvedova AA, et al. Direct evidence for recycling of myeloperoxidase-catalyzed phenoxyl radicals of a vitamin E homologue, 2,2,5,7,8-pentamethyl-6-hydroxy chromane, by ascorbate/dihydrolipoate in living HL-60 cells. *Biochim Biophys Acta* 2003; 1620:72-84.
 10. Gorąca A, Huk-Kolega H, Piechota A, Kleniewska P, Ciejka E, Skibska B. Lipoic acid - biological activity and therapeutic potential. *Pharmacol Rep* 2011; 63:849-58.
 11. Li G, Fu J, Zhao Y, Ji K, Luan T, Zang B. Alpha-lipoic acid exerts anti-inflammatory effects on lipopolysaccharide-stimulated rat mesangial cells via inhibition of nuclear factor kappa B (NF- κ B) signaling pathway. *Inflammation* 2015; 38:510-9.
 12. Shen HH, Lam KK, Cheng PY, et al. Alpha-lipoic acid prevents endotoxemic shock and multiple organ dysfunction syndrome induced by endotoxemia in rats. *Shock* 2015; 43:405-11.
 13. Çimen B, Türközkan N, Unlü A, Erbil MK. Effects of melatonin on 3-nitrotyrosine formation and energy charge ratio in guinea pig kidney in LPS-induced stress. *Cell Biochem Funct* 2005; 23:273-277.
 14. Sung MJ, Kim W, Ahn SY, et al. Protective effect of alpha-lipoic acid in lipopolysaccharide-induced endothelial fractalkine expression. *Circ Res* 2005; 97:880-90.
 15. Ueki M, Taie S, Chujo K, et al. Urinary trypsin inhibitor reduces inflammatory response in kidney induced by lipopolysaccharide. *J Biosci Bioeng* 2007; 104:315-20.
 16. Toth I, Heard SO. Nitric oxide does not mediate lipopolysaccharide-induced myocardial depression in guinea pigs. *Crit Care Med* 1997; 25:684-8.
 17. De Blas Bravo I, Sanz Castro R, López Riquelme N, Tormo Díaz C, Apraiz Goyenaga D. Optimization of the trace element determination by ICP-MS in human blood serum. *J Trace Elem Med Biol* 2007; 21:14-7.
 18. Huk-Kolega H, Ciejka E, Skibska B, Kowalczyk A, Goraca A. [Influence of lipoic acid on the level of TNF-alpha in spleen homogenates]. *Pol Merkuriusz Lekarski* 2014; 36:379-381.
 19. Tanaka Y, Kaibori M, Miki H, et al. Alpha-lipoic acid exerts a liver-protective effect in acute liver injury rats. *J Surg Res* 2015; 193:675-83.
 20. Polat B, Halici Z, Cadirci E, et al. The effect of alpha-lipoic acid in ovariectomy and inflammation-mediated osteoporosis on the skeletal status of rat bone. *Eur J Pharmacol* 2013; 718:469-74.
 21. Ottesen LH, Harry D, Frost M, et al. Increased formation of S-nitrothiols and nitrotyrosine in cirrhotic rats during endotoxemia. *Free Radic Biol Med* 2001; 31:790-8.
 22. Ozdemir S, Toplan S, Ercan M, Bayrak I, Uruluer B, Sunamak O. The effect of β -glucan on trace element levels in intra-abdominal sepsis in rats. *Biol Trace Elem Res* 2009; 132:197-206.
 23. Frisk P, Ola Darnerud P, Friman G, Blomberg J, Ilbäck NG. Sequential trace element changes in serum and blood during a common viral infection in mice. *J Trace Elem Med Biol* 2007; 21:29-36.
 24. Gupta SK, Singh SP, Shukla VK. Copper, zinc, and Cu/Zn ratio in carcinoma of the gallbladder. *J Surg Oncol* 2005; 91: 204-8.
 25. Pompilio A, Ciavardelli D, Crocetta V, et al. *Stenotrophomonas maltophilia* virulence and specific variations in trace elements during acute lung infection: implications in cystic fibrosis. *PLoS One* 2014; 9:88769.
 26. Wang L, Cherayil BJ. Ironing out the wrinkles in host defense: interactions between iron homeostasis and innate immunity. *J Innate Immun* 2009; 1:455-64.
 27. Xia J, Wishart DS. Web-based inference of biological patterns, functions and pathways from metabolomic data using Metabo Analyst. *Nat Protoc* 2011; 6:743-60.
 28. Jakubovics NS, Jenkinson HF. Out of the Iron Age: new insights into the critical role of manganese homeostasis in bacteria. *Microbiology* 2001; 147:1709-18.
 29. Kehl-Fie TE, Skaar EP. Nutritional immunity beyond iron: a role for manganese and zinc. *Curr Opin Chem Biol* 2010; 14:218-24.