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ORIGINAL ARTICLE

INFLUENCE OF TRACE ELEMENTS WITHIN NORMAL PHYSIOLOGICAL RANGE ON NEUTROPHIL BASAL REACTIVE OXYGEN SPECIES PRODUCTION IN THE JAPANESE GENERAL POPULATION

Haruyuki Ito[1,2], Ippei Takahashi[1], Masashi Matsuzaka[1], Sangun Lee[2], Kazuyoshi Itai[3], Shinji Fukui[1,4], Naoko Hamada[1,5], Yuriko Saito[1,6], Takashi Umeda[1] and Shigeyuki Nakaji[1]

Abstract Objective: We investigated the relationship between trace element concentrations and neutrophil basal reactive oxygen species (ROS) production capability among the Japanese general population.

Subjects and methods: Subjects of the current study were 606 participants (225 males and 381 females) who had participated in the Iwaki Health Promotion Project 2005. Body mass index, biochemical blood examination, neutrophil basal ROS production and serum concentrations of trace element (Copper(Cu), Zinc(Zn), Iron(Fe), Selenium (Se)) were measured. Each trace element was divided into 4 quartiles according to the concentration, and the neutrophil basal ROS production of each group was compared and analyzed. Moreover, the relationship between the concentration and neutrophil basal ROS production capability was assessed using a multiple linear regression analysis.

Results: For both males and females, a tendency to low neutrophil ROS production associated with higher Se serum concentration was observed. On the other hand, no significant correlation was observed between the Cu, Zn and Fe concentrations and neutrophil basal ROS production capability.

Conclusion: In conclusion, serum Se relieves oxidative stress through inhibiting neutrophil ROS production within a physiological range. On the other hand, our results suggested that neutrophil ROS production was unaffected by Cu, Zn and Fe concentrations within the normal physiological range.

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Key words: trace elements; selenium; neutrophil; reactive oxygen species; general population.

原　著

生体内必須微量元素が好中球の活性酸素種産生量に及ぼす影響

伊　藤　治　幸[1,2]　高　橋　一　平[1]　松　坂　方　士[1]　李　　相　潤[2]
板　井　一　好[3]　福　井　真　司[1,4]　浜　田　菜穂子[1,5]　齋　藤　百合子[1,6]
梅　田　　孝[1]　中　路　重　之[1]

抄録　近年，必須微量元素の欠乏が酸化ストレスを介して生活習慣病や老化に影響を与えている可能性が指摘されている．そこで，本研究では，地域一般住民606名(男性225名，女性381名)を対象として，生理的な濃度における生体内必須微量元素と好中球の平常時活性酸素種産生量(Basal ROS 産生量)の関係について疫学的に調査・検討した．その結果，男女共に，Se の血中濃度が高い人ほど好中球の Basal ROS 産生量が少ない傾向がみられた．一方，Cu，Zn，Fe については好中球 Basal ROS 産生量と関連はみられなかった．したがって，生理的な濃度範囲内であっても Se は好中球の ROS 産生に対して抑制効果を示し，好中球による酸化ストレスを抑えている可能性が示唆された．一方，Cu，Zn，Fe については，生理的な濃度範囲内であれば好中球の ROS 産生量に影響を与えない可能性が示唆された．

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[1] Department of Social Medicine, Hirosaki University Graduate School of Medicine
[2] Department of Health Science, Aomori University of Health and Welfare
[3] Department of Hygiene and Preventive Medicine, Iwate medical University
[4] Shokei Gakuin University
[5] Nursing School, National Defense Medical College
[6] Department of Plastic and Reconstructive Surgery, Hirosaki University of Graduate School of Medicine
Correspondence: I. Takahashi
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[1] 弘前大学大学院医学研究科社会医学講座
[2] 青森県立保健大学健康科学部
[3] 岩手医科大学医学部衛生学公衆衛生学講座
[4] 尚絅学院大学
[5] 防衛医科大学校高等看護学院
[6] 弘前大学大学院医学研究科形成外科学講座
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Introduction

In recent years, trace elements have been found to be heavily involved in lifestyle related diseases and ageing. For example, the serum concentration of copper (Cu) was reported to associate with age-related diseases[1-2]. Individuals with low zinc (Zn) intakes and low iron (Fe) concentrations were also reported to have higher risks of lifestyle related diseases[2-3]. Moreover, the risks of cancer and infectious diseases have shown to be increased with low selenium (Se) concentrations[4-5]. Recently, the involvement of oxidative stress has been predicted to be a part of these findings, although only a small number of studies have been carried.

To date, the majority of studies on the relationship between oxidative stress and trace elements have focused on the effect of trace elements on the scavenging system of reactive oxygen species (ROS), which is the cause of oxidative stress[6-8]. Several trace elements are involved in the antioxidant enzyme activity depend on ROS scavenging activity[9], and there have been many reports on the shortage of trace elements causing the reduction of ROS scavenging activity[10-12]. However, recent studies showed that these trace elements (e.g. Cu, Zn, Fe and Se) were also related to the ROS generating system. In other words, some reports have suggested that Cu, Zn and Fe activates and Se suppresses production of ROS in the NADPH oxidase system[13-17]. However, these reports have focused on the NADPH oxidase in nerve cells of the brain and renal cells. The role of NADPH oxidase in oxidative stress in the body in general remains unclear.

One of the main causes of physical oxidative stress is the ROS production mechanism (e.g. NADPH oxidase system) of immune cells such as neutrophils [18]. Neutrophils are one of the central elements of innate immunity, playing an important role in the first line of defence against foreign substances, including microorganisms[19]. Neutrophils engulf microorganisms [phagocytic activity (PA)] and produce ROS in order to kill them. However, excess production of ROS has been suggested to cause oxidative damage in normal tissues[20-21]. In particular, neutrophil ROS production during the normal state (basal ROS production) is excessive for a long time, it may produce physical oxidative stress[22] leading to many health disorders. Therefore, neutrophil basal ROS production is usually maintained at low levels[23].

In terms of the effects of Cu, Zn, Fe and Se on neutrophil ROS production, only the relationship between the Se concentration and ROS production has been investigated, and the individuals with low Se concentrations have been reported to have higher neutrophil ROS production than the others[24]. However, some reports suggest that there are interrelations among these elements[25]. The reduction of antioxidant enzyme activity due to the imbalance in these trace elements have also been reported[26-27], and a reduction in serum Fe and SOD activity may follow on from an excessive intake of Zn. Also, there are significant correlations among these elements with each other[28]. Thus, studies on trace elements are required to control the interaction of the elements on each other. However, a study on the relationship between Cu, Zn, Fe and Se, and ROS production, which could eliminate the existence of any interrelation among the trace elements, has not been conducted. Alternatively, studies which investigated the effects of trace elements in terms of the neutrophil response against foreign substances have already shown the possibility of Cu, Zn, Fe and Se shortages leading to reduced neutrophil ROS production[29-32].

In the present investigation, the relationship between the levels of Cu, Zn, Fe, and Se and neutrophil basal ROS production in the general population was assessed epidemiologically, and their relationships were analysed after removing the influence which trace elements have on one another.

Cu, Zn, Fe and Se are trace elements which are indispensable to humans. They cannot be synthesized within the body, and must be taken externally. Therefore, the aim of this study was to devise the outline of an eating regime to prevent oxidative stress-related illness through the regulation of trace element intake.

The present study had the following 2 advantages.

1) The present study measured not only the serum concentration of Se, but also Cu, Zn and Fe, and assessed the findings in such a way that could take the influence of the interrelationship among the trace elements into consideration.

2) Unlike many previous studies, this study assessed the ROS production in the absence of stimulants (basal ROS), which is the more common state of human neutrophils in their normal state. During almost every daylight and night hour, circulating neutrophils exist in the blood without any stimulation by foreign substances (basal ROS). Therefore, basal ROS is very important for understanding the longtime accumulative effect against the physiologically body condition of many health disorders including chronic lifestyle diseases. Therefore, we were able to examine the relationship between the trace elements and the ROS production of subjects under normal conditions.

Subjects and Methods

Subjects

The subjects of this study were selected from 1,067 adults who participated in the Iwaki Health Promotion Project 2005 and gave their consent to partake in the study after having the details of the study explained to them. A total of 606 participants (225 males and 381 females) who met all the criteria, filled in the questionnaire correctly and had no major organ diseases (cancer, cardiac diseases, stroke, thyroid or renal disease, diabetes or inflammatory disease)[4, 5, 33-37], were enrolled as the subjects of the present study.

The current study was approved by the Hirosaki University Graduate School of Medicine Medical Research Ethics Committee. Prior to the investigation, the purpose, procedure and details of the study were thoroughly explained to all subjects and written consent was obtained from each of them.

Questionnaire

The questionnaire was given to each subject to complete, and an individual interview was carried out on the day of serum collection. The assessment criteria of subjects included sex, age, habits as to smoking, drinking and physical exercise, current and previous medical history, and whether the subject had ever taken non-steroidal anti-inflammatory drugs (NSAIDs). In order to determine the physique of subjects, body mass index (BMI) was calculated based on their height and weight.

Measurement of serum and plasma chemical parameters

The serum collection was carried out in the morning under a fasting state. Subjects were asked to remain in a seated position and samples were taken from their median cubital vein. The total white blood cell count and the neutrophil count were determined using an automated blood analyser SE-9000 (Sysmex, Kobe, Japan). The concentration of each trace element (Cu, Zn, Fe and Se) was measured with Inductively Coupled Plasma Mass Spectrometry (ICP-MS), based on the abundance of the element. The unit of each trace element was written by μg/dl.

Neutrophil Basal ROS production

The ROS production of peripheral blood neutrophils was determined with the FACScan system (Becton Dickinson, San Jose, CA) using two-color flow cytometry. Hydroethidine (HE; 44.4 μmol/L, Polyscience Inc., Warrington, PA) was used as an indicator for ROS production capability. Briefly, 100 μL of heparinized whole blood was mixed with 22 μL of HE (final concentration, f.c. 8 μmol/L) and incubated at 37°C for 5 min. After incubation, Lyse and Fix (IMMUNOTECH, Marseille, France) was added to lyse the erythrocytes and to fix the samples.

The samples were washed twice in phosphate-buffered saline with sodium azide, and the fluorescence intensity (FI) in activated neutrophils was measured with the FACScan. The percentages of activated neutrophils were calculated. The cumulative fluorescence intensity (CFI), the sum of the values of FI multiplied by the percentage of positive cells, was used as the quantitative index.

Statistical analysis

Descriptive analyses were conducted statistically on subjects' physical characteristics, lifestyles, concentration of each trace element and neutrophil basal ROS production. The mean concentration ± standard deviation (SD) or adjusted mean ± standard Error (SE) and 95% confidence interval were calculated for each trace element, and the Pearson's correlation coefficient was calculated to assess the relationship between each of the trace element in the serum. Moreover, subjects were categorized into quartiles based on the levels of the trace element serum concentrations. In order to examine the effect of each trace element on neutrophil basal ROS production, an analysis of covariance was conducted with the neutrophil ROS production for each quartile group as the objective variable. Additionally, a multiple linear regression analysis was conducted to examine the linear effect of trace element concentrations on the neutrophil basal ROS production. Age, BMI and habits of smoking, drinking, physical exercise and other trace elements were chosen as the correction factor (confounding factor). SPSS12.0J for Windows (SPSS Japan Inc.) was used for the analyses and $p < 0.05$ was considered statistically significant.

Results

Characteristics and lifestyles of subjects are listed in Table 1. Values were presented as a mean ± SD and as a percentage for males and females. The proportion of current smoker and current drinker and exercise frequency were higher in male than those in female (p=0.00 all).

The mean concentration of each serum component and trace elements and neutrophil basal ROS production is shown in Table 2. Leukocyte and neutrophil counts were higher in male than those in female (p=0.00 all), although value of basal ROS production capability was lower in male than that those in female (p=0.03). The concentrations of serum Zn, Fe and Se were higher in male than in female (p=0.02, p=0.00, p=0.00, respectively).

In terms of the correlation between each trace element presented as Pearson's correlation coefficients, a significant correlation was observed between Zn and Fe ($p<0.05$) for males (Table 3). For females, significant correlations were observed between Cu and Zn ($p<0.01$), Cu and Se ($p<0.01$), Zn and Fe ($p<0.05$) and Zn and Se ($p<0.01$).

Each trace element was divided into 4 quartiles according to the concentration. The range of concentration quartiles for each element in males and females is shown in Table 4.

Table 5 shows the comparison of the neutrophil basal ROS production values among serum element concentrations quartile using the analysis of covariance, after adjusted by three

Table 1 Characteristics of subjects.

	Male (n = 225)	Female (n = 381)	p-value
Age (years)	50.5 ± 14.5	52.2 ± 14.0	0.16[a]
BMI (kg/m^2)	23.1 ± 2.9	22.4 ± 3.0	0.01[a]
Smoking habit			0.00[b]
Never smoker	59 (26.2)	308 (80.8)	
Currently smoker			
<5 cigarettes/day	5 (2.2)	13 (3.4)	
5-9 cigarettes/day	16 (7.1)	22 (5.8)	
10-19 cigarettes/day	60 (26.7)	24 (6.3)	
20-29 cigarettes/day	64 (28.4)	14 (3.7)	
>30 cigarettes/day	21 (9.3)	0 (0.0)	
Drinking habit			0.00[b]
Never drinker	57 (25.3)	278 (73.0)	
Currently drinker			
<20 g/day	34 (15.1)	67 (17.6)	
20-39 g/day	41 (18.3)	17 (4.4)	
>40 g/day	93 (41.3)	19 (5.0)	
Physical activity			0.00[b]
None	170 (75.5)	333 (87.4)	
Active			
1/W	18 (8.0)	11 (2.9)	
2-3/W	13 (5.8)	13 (3.4)	
4-5/W	11 (4.9)	9 (2.4)	
Everyday	13 (5.8)	15 (3.9)	

Mean ± Standard Deviation (SD) or n (%), BMI: body mass index. [a]: unpaired t-test, [b]: chi square test.

Table 2 Measurements of serum and plasma chemical parameters.

	Male (n = 225)	Female (n = 381)	p-value
Total leukocyte counts (/μL)	5729.3 ± 1612.2	4923.3 ± 1432.8	0.00
Neutrophil cell counts (/μL)	3292.9 ± 1262.0	2813.1 ± 1184.5	0.00
basal ROS producution (CFI)	187.7 ± 330.1	307.7 ± 544.6	0.03
Cu (μg/dL)	98.6 ± 66.6	98.5 ± 22.4	0.98
Zn (μg/dL)	102.9 ± 20.3	99.3 ± 18.9	0.02
Fe (μg/dL)	120.0 ± 43.4	99.6 ± 38.6	0.00
Se (μg/dL)	24.4 ± 3.6	23.3 ± 4.2	0.00

Mean ± Standard Deviation (SD), Statistical significance: p-value using the unpaired t-test, CFI; cumulative fluorescent intensity ($\times 10^2$).

other trace element concentrations, age, BMI, smoking, drinking and physical exercise habits. As a result, ROS production was shown to decrease with the increased concentration of Se in both males and females (for both males and females p = 0.03), and such tendency was not observed with other trace elements (Table 5).

Moreover, a multiple linear regression analysis was conducted after removing each influencing factor (age, BMI, amount of alcohol intake, amount of cigarette smoking, exercise habits and other trace elements), in order to examine the linear effect of each trace element concentration on neutrophil basal ROS production. In both males and females, a negative significant correlation was seen only for Se (for both males and females p = 0.00) (Table 6). On the other hand, no significant correlation was

Table 3 Pearson's correlation coefficients among serum trace element concentrations.

		Zn	Fe	Se
Male n = 225	Cu	-0.02	-0.13	0.08
	Zn	-	0.13*	0.01
	Fe	-	-	0.07
		Zn	Fe	Se
Female n = 381	Cu	0.33**	0.01	0.15**
	Zn	-	0.23*	0.23**
	Fe	-	-	0.08

*: p<0.05, **: p<0.01

Table 4 Serum trace element concentrations in each quartile.

		Q1	Q2	Q3	Q4
Male n = 225	Cu (μg/dL)	43.2-83.6	83.6-93.6	93.7-104.3	>104.4
	Zn (μg/dL)	67.3-89.1	89.2-100.7	100.8-112.1	>112.2
	Fe (μg/dL)	25.0-87.0	87.1-119.0	119.1-145.5	>145.6
	Se (μg/dL)	17.1-21.9	22.0-24.0	24.1-26.5	>26.6
		Q1	Q2	Q3	Q4
Female n = 381	Cu (μg/dL)	28.5-88.6	88.7-97.2	97.3-108.1	>108.2
	Zn (μg/dL)	53.6-88.5	88.6-97.2	97.3-107.3	>107.4
	Fe (μg/dL)	16.0-76.5	76.6-96.0	96.1-122.0	>122.1
	Se (μg/dL)	10.0-20.8	20.9-22.9	23.0-25.6	>25.7

Table 5 Values of ROS production capability according to serum trace elements concentration.

		Q1	Q2	Q3	Q4	p-value
Male n = 225	Cu (μg/dL)	124.0 ± 42.9	243.0 ± 42.4	216.9 ± 42.5	165.8 ± 43.2	0.19
	Zn (μg/dL)	179.3 ± 42.8	228.4 ± 41.6	176.0 ± 44.9	163.5 ± 43.8	0.71
	Fe (μg/dL)	224.8 ± 43.2	187.1 ± 42.1	153.2 ± 42.3	186.0 ± 43.7	0.71
	Se (μg/dL)	282.7 ± 42.3	187.1 ± 41.4	175.2 ± 42.0	105.5 ± 41.9	0.03
		Q1	Q2	Q3	Q4	p-value
Female n = 381	Cu (μg/dL)	313.3 ± 56.9	221.9 ± 55.0	397.7 ± 56.1	300.3 ± 56.2	0.17
	Zn (μg/dL)	345.7 ± 55.9	333.3 ± 55.6	220.6 ± 55.5	333.0 ± 57.0	0.34
	Fe (μg/dL)	342.0 ± 57.1	385.8 ± 55.7	261.1 ± 56.0	241.2 ± 56.9	0.24
	Se (μg/dL)	442.1 ± 55.3	277.2 ± 55.0	302.5 ± 55.3	209.0 ± 55.4	0.03

Adjusted Mean ± Standard Error (SE); Values are adjusted for age, BMI, amount of alcohol intake, amount of cigarette smoking, exercise habits and other three trace elements.

observed with neutrophil basal ROS production and concentrations of Cu, Zn or Fe.

Discussion

Although some of the essential trace elements have been reported to influence oxidative stress including neutrophil ROS-related stress, the mechanisms are less clear. Therefore, in the present study we investigated the relationship between neutrophil basal ROS production and trace elements among the general population.

The subjects of this study were representative

Table 6 Multiple regression analysis of relationship between serum trace element concentrations and neutrophil basal ROS production.

	Male (n=225)			Female (n=381)		
	β	p-value	R^2	β	p-value	R^2
basal ROS production (CFI)						
Cu	-0.05	0.44	0.08	-0.08	0.10	0.08
Zn	0.03	0.59	0.08	0.02	0.57	0.04
Fe	-0.03	0.61	0.08	-0.03	0.52	0.04
Se	-0.18	0.00	0.11	-0.13	0.00	0.22

β: standardized partial regression coefficient; p-value: significance probability; R^2: coefficient of determination; ROS: reactive oxygen species; CFI: cumulative fluorescence intensity; Values are adjusted for age, BMI, amount of alcohol intake, amount of cigarette smoking, exercise habits and other three trace elements.

Table 7 Reference values for Cu, Zn, Fe, Se concentrations in the general population.

	Dimension	Area	Mean ± SD (95%Cl)	Reference
Cu	μg/dL	Japan	98.5 ± 44.2 (95.3 - 102.1)	This study
	μg/dL	Portugal	132.0 ± 50.0	38
	mg/L	Canary Islands	110.0 ± 25.0*	39
	μmol/L	Iran	94.3 ± 21.0*	40
Zn	μg/dL	Japan	106.4 ± 19.5 (99.9 - 102.2)	This study
	μg/dL	Portugal	99.0 ± 17.0	38
	mg/L	Canary Islands	116.0 ± 52.0*	39
	μmol/L	Iran	76.5 ± 12.4*	40
Fe	μg/dL	Japan	107.2 ± 41.6 (103.9 - 110.5)	This study
	μg/dL	Korea	104.1 ± 34.9	41
Se	μg/dL	Japan	23.8 ± 40.0 (23.4 - 24.1)	This study
	ng/mL	South Dakota, USA	25.6 ± 3.6*†	42
	nmol/L	Kikwit, DR Congo	20.2 ± 2.7*	43

Cl: confidence interval; *: change for μg/dL; †: mean ± SE (standard error).

adults from the general population, who had no current or previous disease history that might have affected the trace element concentrations. Overall, trace element concentrations of the subjects were found to be similar to those from the previous general population-based studies[38-43] (Table 7). Therefore, the concentrations of each element in the subjects of this study were likely to be within the normal physiological range.

Moreover, significant correlations among the 4 trace elements were observed in both males and females, in a similar manner as in the previous study[28], suggesting the interaction of the elements. This finding shows the importance of removing the interrelation of trace elements on one another equalized.

Many of the previous studies have focused on the effects of Cu, Zn and Fe concentrations on the ROS production system in case they are not within the normal physiological ranges. The excess of these trace elements may lead to production of ROS, an excitatory effect on

NADPH oxidase[14-15], and an inhibitory effect when they are deficient[16]. In an experimental study, an excess amount of Zn administered to cultured renal epithelial cells leads to increase ROS and which is mediated by NADPH oxidase, and may trigger cytopathy in some cases[15]. However, the NADPH oxidase activation mechanism via these trace element is not well understood. Although ROS production by neutrophils is mediated by NADPH oxidase, the results obtained in the current study showed no correlations between Cu, Zn and Fe and neutrophil basal ROS production. In other words, this study suggested that Cu, Zn and Fe concentrations within normal physiological ranges were unlikely to affect the basal neutrophil ROS production.

On the other hand, the present study showed a significant relationship between Se and neutrophil basal ROS production, suggesting that the higher the Se concentration in serum, the lower was the neutrophil basal ROS production. Selenium has been suggested to have an inhibitory effect on NADPH oxidase, which is the mechanism of ROS production[17] in the membrane of the neutrophil. According to this fact, basal ROS production of those with high Se concentration is considered to be low due to inhibition of the ROS production system. Moreover, an inhibition of foreign substance induced ROS production by neutrophils has been reported through the ingestion of food containing large amounts of Se[44]. In other words, Se may exert an inhibitory effect on ROS production in the normal state or upon exposure to a foreign substance (excited state), even when the interaction of trace elements is removed and its concentration is within the normal physiological range. Previously, responses of neutrophil basal ROS production[24] and foreign substance induced ROS production by neutrophils have both been suggested to cause oxidative stress[20-21]. Thus, Se was suggested to be able to suppress the oxidative stress caused by neutrophil-mediated ROS.

For many years, the involvement of oxidative stress has been suggested to be the cause of various diseases[45-47]. On the other hand, the current study suggested that an adequate daily Se intake may suppress the basal ROS production by neutrophils even when it is within the normal physiological range, and could thus possibly prevent chronic oxidative stress. Therefore, regulating the intake of certain trace elements may be one possible method to prevent oxidative stress-related diseases.

In previous study, smoking is known to cause systemic oxidative stress[48]. In the present study, a significant correlation was observed between the concentration of trace element and neutrophil basal ROS production in smokers (not shown data). As a result, only for Se, a negative correlation was observed in both male and female smokers (males β = -0.18, p = 0.04 and females β = -0.14, p = 0.05). Therefore, Se intake was suggested to suppress the oxidative stress by neutrophil-mediated ROS in smokers. In other words, an adequate daily Se intake may suppress the oxidative stress in smokers.

However, no investigation into NADPH oxidase activation was carried out in the present study. Moreover, the effect of trace elements on NADPH oxidase was thought to be caused by the amount of those elements, and this was considered to be a limitation of this study.

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