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Original article

THE EFFECT OF ZINC SUPPLEMENTATION ON POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS OF ELDER HYPERTENSIVE PATIENTS AND HEALTHY YOUNG VOLUNTEERS

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Abstract

Zinc supplementation can stimulate polymorphonuclear leukocyte (PMN) functions (phagocytosis and intracellular killing activity) in older hypertensive patients and healthy young volunteers. The aim of this study is to investigate the effect of zinc supplementation on polymorphonuclear leukocyte (PMN) functions (phagocytosis and intracellular killing activity) in vitro in 13 older hypertensive patients and compare these functions with those of 10 healthy young volunteers. PMNs were isolated by ficoll-hypaque gradient centrifugation method from venous blood with EDTA (0.1g/ml).Phagocytosis and intracellular killing activity were assayed by modifying Alexander's method. The subjects were given zinc supplementation 22mg/daily/30 days. Serum zinc levels were measured by flame atomic absorption spectrometer. The serum zinc levels and the PMN's intracellular killing activities of older hypertensive patients before zinc supplementation were significantly low when compared with that of healthy young volunteers ($p<0.001$, $p=0.001$ respectively). After zinc supplementation the PMN's phagocytic and intracellular killing activities of older hypertensive patients were significantly lower than those of healthy young volunteers ($p<0.001$, $p<0.05$ respectively). However the serum zinc level of the elderly increased after zinc supplementation when compared with that before supplementation . The serum zinc levels and PMN's phagocytic activity of healthy young volunteers significantly increased after zinc supplementation ($p<0.05$). Consequently, the serum zinc levels of the older hypertensive patients and young volunteers increased after zinc supplementation. Adequate zinc supplementation may improve PMN functions both in older hypertensive patients and healthy young volunteers.

Key Words: *Polymorphonuclear leukocyte functions, Phagocytosis, Intracellular killing, Older hypertensive patients, Zinc supplementation.*

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Çinko Desteğinin Yaşlı Hipertansiyonlu Hasta ve Sağlıklı Gençlerin Polimorf Nüveli Lökosit Fonksiyonları Üzerine Etkisi

Çinko desteği yaşlı hipertansiyonlu hastaların ve sağlıklı gençlerin polimorf nüveli lökosit (PNL) fonksiyonlarını (fagositoz ve hücre içi öldürme aktivitesi) stimüle edebilir. Bu çalışmanın amacı çinko desteğinin 13 yaşlı hipertansiyonlu hasta ile 10 sağlıklı gencin PNL fonksiyonları üzerine olan etkilerini in vitro koşullarda araştırmaktır. PNL'ler EDTA'lı venöz kandan Ficoll-Gradient santrifüj yöntemi ile ayrılmıştır. Fagositoz ve hücre içi öldürme aktivitesi tayininde Alexander ve arkadaşlarının yöntemi modifiye edilerek kullanılmıştır. Yaşlı hipertansiyonlu hasta ve sağlıklı kişilere 30 gün boyunca 22mg/gün/oral olarak çinko desteği verilmiştir. Serum çinko düzeyleri atomik absorpsiyon spektrofotometresi ile belirlenmiştir. Çinko desteği öncesi yaşlı hipertansiyonlu hastaların serum çinko düzeyleri ve PNL'in hücre içi öldürme aktivitesi sağlıklı gençlere göre anlamlı olarak düşük bulunmuştur ($p<0.001$, $p=0.001$ sırasıyla). Çinko desteği sonrası yaşlı hipertansiyonlu hastaların PNL'lerin fagositik aktivitesi ve hücre içi öldürme aktivitesi sağlıklı gençlere göre anlamlı olarak düşük bulunmuştur ($p<0.001$, $p<0.05$ sırasıyla). Ancak çinko desteği sonrası yaşlı hipertansiyonlu hastaların serum çinko düzeyi destek öncesine göre artmıştır. Çinko desteği sonrası sağlıklı gençlerin serum çinko düzeyleri ile PNL'lerin fagositik aktivitesi anlamlı olarak artmıştır ($p<0.05$). Sonuç olarak, çinko desteği sonrası yaşlı hipertansiyonlu hasta ve sağlıklı gençlerin serum çinko düzeyleri artmıştır. Doğru dozda yapılan çinko desteği yaşlı hasta ve sağlıklı gençlerin PNL'lerinin her 2 fonksiyonunu anlamlı olarak artırabilir .

Anahtar Kelimeler: *Polimorf nüveli lökosit fonksiyonları, Fagositoz, Hücre içi öldürme aktivitesi, Yaşlı hipertansiyonlu hasta, Çinko desteği.*

INTRODUCTION

Due to age related changes of the immune system, the rate of morbitidy and mortality from infectious diseases in older hypertensive people is higher than that of younger adults (1,2) . Neutrophil granulocytes (PMNs) are the first cells to migrate into the side of infection, forming the host's primary line of defense against infection. Since the functions of PMNs are phagocytosis and killing of the invading microorganisms, an age-related decline in PMN functions may be responsible for this increased susceptibility to infections in the older populations. Although the number of circulating neutrophils remains unaltered in the older people compared with young controls, PMN's phagocytic and intracellular killing activities have been reported to be impaired (1). Previous studies on older people showed that there is an age related increase in plasma membrane viscosity, which may be related to the diminished production of superoxide anion (O_2^-) and may result in impaired neutrophil function (3). Using a variety of stimuli, age related decreases have been demonstrated in phagocytic ability (2) and intracellular killing activity (1,4,5), superoxide production (3,6) neutrophil adhesion, receptor mediated superoxide responses (6) and chemotaxis (7).

In addition, the findings suggest that increased intracellular Ca^{+2} concentrations in resting neutrophils and/or a reduced hexose uptake result in reduced phagocytic ability and decreased bactericidal activity of neutrophils in the older people (5).Protein-energy malnutrition is associated with the significant impairment of phagocyte functions, cell mediated immunity complement system and secretory immunoglobulin response. Deficiency of a single nutrient also results in altered immune response, which is observed even when the deficiency state is relatively mild (8).

Studies performed around the world prove that zinc deficiency has become a nutritional problem affecting both developed and developing countries (8). The clinical manifestations of zinc deficiency are growth retardation, susceptibility to infection caused by fungi, viruses and bacteria, thymic atrophy, reduced lymphocyte proliferative response to mitogens, decreased

ratio of helper and supressor T lymphocytes, decreased NK cell activity, hypogonadism, anorexia and diarrhea (9,10).

Several studies were performed in vivo in animals and human in order to find the relation between zinc levels and immune functions and at the same time to elucidate the role that zinc plays in molecular level (9). Some authors point out that the zinc levels of lymphocytes and granulocytes in the older population is deficient when compared to young controls (10).

The aim of our study is to assess the effect of serum zinc levels on polymorphonuclear leukocyte (PMNs) functions (phagocytosis and intracellular killing activity) in older hypertensive patients and healthy young volunteers and at the same time to compare PMN functions with serum zinc levels in these two groups.

EXPERIMENTAL

Subjects

Twenty-three subjects were included in this study: 13 older hypertensive patients with a mean age of 71 years, range 60-86. The older hypertensive patients had hypertension. The control group consisted of 10 healthy young volunteers with a mean age of 25, years range 20-31, all of whom were drug-free individuals.

Blood samples were obtained from 13 older hypertensive patients who had hypertension and were given 5 mg enalapril maleate during their treatment (Nobel Pharmaceutical Inc., Istanbul,Turkey). They were hospitalized in the Department of Internal Medicine, Istanbul Faculty of Medicine, University of Istanbul, in Turkey.

Zinc

Thirteen older hypertensive patients and 10 healthy young volunteers were given zinc supplementation for 30 days, 22mg zinc picolinate/day/oral (Solgar, Leonia, New Jersey, USA).

Isolating PMNs

Phagocytic and intracellular killing activities of the PMNs were assayed using a modification of Alexander's method (11). In the modified method, Ficoll was used instead of dextran and PMNs were counted by microscope instead of standard pour plate technique. The PMNs were isolated from the venous blood by the Ficoll-Hypaque gradient centrifugation method (12,13,14). Twenty milliliters of venous blood was drawn from both older hypertensive patients and the control subjects into 0.1 g/ml ethylenediaminetetraacetic acid. The blood was centrifuged at 2500 rpm for 30 min, the buffy-coat layer was removed, added to Ficoll-Hypaque plus Polymorphprep solution and centrifuged at 3000 rpm for 30 min. The PMN layer was removed, washed 3 times, and cell suspensions were adjusted to 1 $x10^7$ cells/ ml in Hanks's Buffered Salt Solution (HBSS). The PMNs were found to be >99% viable by trypan blue exclusion (0,5%, in 0,9 percent saline solution) (12,14).

Measurement of Phagocytosis and Intracellular Killing Activity

A clinical strain of *Candida albicans* was used to determine the phagocytic and intracellular killing activity of PMNs. *C. albicans* viability was assayed as greater than 98% by methylene blue staining. PMNs suspended in HBSS were incubated at 37°C for 30 min in a shaking incubator. *C. albicans* (in another tube) was suspended in HBSS, and then an aliquoat of sterile human serum (1:4) was added (in order to opsonize it) followed by incubation at 37°C for 30 min. Yeast cells were then added to the PMN tube, and the final mixture contained 5×10^6 PMNs/ml and 5×10^6 yeast cells/ml. Dead yeast cells were determined after adding 0.01% methylene blue stain (1:1 ratio) in the last 5 min of the incubation. The phagocytic activity was

determined by the percentage of PMNs that had phagocytosed yeasts cells. The intracellular activity was determined by the percentage of PMNs that included killed yeast cells as described previously (12,14).

Measurement of Serum Zinc Levels

Blood taken in sterile silicon tubes was centrifuged at 2500 rpm for 20 minutes and then the serum was taken into a sterile tube and was kept at –20°C for serum zinc determination. Before serum zinc measurement the serums kept at –20°C were taken into room temperature and then they were diluted with trichloroacetic acide (TCA) (1:4). In order to precipitate; the serum was left 10 minutes at room temperature prior to analysing by flame atomic absorption spectrometer. The mixture of serum and TCA was centrifuged at 3000 rpm for 5 minutes and clear supernatant was analysed (15).

Statistics

Data were entered onto Excel database and analysed using SPSS version 10.0. The results were expressed by means ±SD. Differences between groups were calculated by using Wilcoxon Signed Ranks Test and Mann-Whitney Test. *P* values less or equal to 0.05 were considered to be statistically significant.

RESULTS

Table 1 shows the relationship between the serum zinc levels and PMN functions (phagocytosis and intracellular killing activity) in older hypertensive patients (n=13) and healthy young volunteers (n=10) before zinc supplementation .

The both PMN functions (phagocytosis and intracellular killing activity) of 10 healthy young volunteers whose serum zinc levels were between 1.07-3.76 ppm were found to be normal (100%) before zinc supplementation .

In the second part of Table 1 the older hypertensive patients were divided into 3 groups according to their serum zinc levels: 10 older hypertensive patients had serum zinc levels below the serum zinc range of healthy young volunteers (0.49-1.06 ppm), 2 older hypertensive patients had the same serum zinc range as that of healthy young volunteers (1.07-3.76 ppm), 1 older hypertensive patients had serum zinc level above the serum zinc range of healthy young volunteers (5.92 ppm).

Table 1. Relationship between the serum zinc levels and PMN functions (phagocytosis and intracellular killing activity) in older patients (n=13) and healthy young volunteers (n=10) before zinc supplementation.

Healthy young volunteers (n=10)				
	Serum zinc levels (1.07-3.76 ppm) (n=10)			
	PMN functions			
	Phagocytosis (%)*		Intracellular killing activity (%)**	
	Normal	Low	Normal	Low
Donor	10	0	10	0
%	100	0	100	0

Older patients (n=13)												
	Serum zinc levels (0.49-1.06ppm) (n=10)				Serum zinc levels(1.07-3.76 ppm) (n=2)				Serum Zinc Level (5.92) (n=1)			
	PMN functions				PMN functions				PMN functions			
	Phagocytosis (%)*		Intracellular killing activity (%)**		Phagocytosis (%)*		Intracellular killing activity (%)**		Phagocytosis (%)*		Intracellular killing activity (%)**	
	Normal	Low	Normal	Low	Normal	Low	Normal	Low	Normal	Low	Normal	Low
Donor	6	4	5	5	2	0	1	1	1	0	1	0
%	60	40	50	50	100	0	50	50	100	0	100	0

* Phagocytic activity above 30 is accepted as normal.
** Intracellular killing activity above 4 is accepted as normal.

As it is seen from Table 1 the PMN's phagocytic activities of 10 older hypertensive patients whose serum zinc levels were between 0.49-1.06 ppm, were found to be low in 4 patients (40%) and to be normal in 6 patients (60%). The intracellular killing activities of the same group was found to be low in 5 patients (50%) and normal in 5 patients (50%) in the same group.

The PMN's phagocytic activities of 2 older hypertensive patients whose serum zinc levels were between 1.07-3.76 ppm were found to be normal (100%). The PMNs' intracellular killing activities were found to be normal in 1 patient (50%) and low in 1 patient (50%) in the same group.

Both PMN activities of 1 older hypertensive patient whose serum zinc level was 5.92 ppm were found to be normal (100%).

Table 2 shows the comparison of PMN functions (phagocytosis and intracellular killing activity) and serum zinc levels in older hypertensive patients (n=13) and healthy young volunteers (n=10) before zinc supplementation.

Table 2. Comparison of PMN functions (phagocytosis and intracellular killing activity) and serum zinc levels in older hypertensive patients (n=13) and healthy young volunteers (n=10) before zinc supplementation.

		PMN Functions	
Group	Serum zinc level (ppm)	Phagocytosis (%)	Intracellular killing (%)
Older patients n=13	1.09±0.97	48.46±17.18	4.46±4.31
Healthy young volunteers n=10	1.92±0.73*	45.00±7.49	9.50±2.59**

Differences between groups were calculated by using Wilcoxon Signed Ranks Test and the data shown are by means ± SD, *$p<0.001$, **$p=0.001$.

As it is seen from Table 2, the serum zinc levels of older hypertensive patients before zinc supplementation were significantly low when compared with that of healthy young volunteers ($p<0.001$).

There was no significant difference between the PMN's phagocytic activity of older hypertensive patients and healthy young volunteers .

On the other hand, the PMNs' intracellular killing activities of older hypertensive patients were significantly lower from that of healthy young volunteers ($p=0.001$).

Table 3 shows the comparison of PMN functions (phagocytosis and intracellular killing activity) and serum zinc levels in older hypertensive patients (n=13) and healthy young volunteers (n=10) after zinc supplementation.

Table 3. Comparison of PMN functions (phagocytosis and intracellular killing activity) and serum zinc levels in older hypertensive patients (n=13) and healthy young volunteers (n=10) after zinc supplementation.

		PMN Functions	
Group	Serum zinc level (ppm)	Phagocytosis (%)	Intracellular killing (%)
Older patients n=13	5.71±13.28	41.54±11.45	6.69±4.31
Healthy young volunteers n=10	3.30±1.21**	60.50±5.98*	10.90±4.88**

Differences between groups were calculated by using Wilcoxon Signed Ranks Test and the data shown are by means ± SD, *$p<0.001$, **$p<0.05$.

As it is seen from Table 3 the serum zinc levels of healthy young volunteers were significantly lower than those of older hypertensive patients ($p<0.05$).

On the other hand, the PMN's phagocytic and intracellular killing activities of older hypertensive patients were significantly lower than those of healthy young volunteers ($p<0.001$, $p<0.05$ respectively).

Table 4 shows PMN functions (phagocytosis and intracellular killing activity) and serum zinc levels in older hypertensive patients (n=13) before and after zinc supplementation.

Table 4. PMN functions (phagocytosis and intracellular killing activity) and serum zinc levels in older patients (n=13) before and after zinc supplementation.

		PMN Functions	
Zinc supplementation	Serum zinc level (ppm)	Phagocytosis (%)	Intracellular killing (%)
Before	1.09±0.97	48.46±17.18	4.46±4.31
After	5.71±13.28	40.02±11.23	6.64±4.14

Differences between groups were calculated by using Wilcoxon Signed Ranks Test and the data shown are by means ± SD, $p>0.05$.

As it is seen from table 4 there was no statistically significant difference between the serum zinc levels, PMN's phagocytic and intracellular killing activity of older hypertensive patients before and after zinc supplementation ($p>0.05$).

Table 5 shows the PMN functions (phagocytosis and intracellular killing activity) and serum zinc levels in healthy young volunteers (n=10) before and after zinc supplementation.

Table 5. PMN functions (phagocytosis and intracellular killing activity) and serum zinc levels in healthy young volunteers (n=10) before and after zinc supplementation.

		PMN Functions	
Zinc supplementation	Serum zinc level (ppm)	Phagocytosis (%)	Intracellular killing (%)
Before	1.92±0.73	45.00±7.49	9.50±2.59
After	3.30±1.21*	60.50±5.98*	10.90±4.88

Differences between groups were calculated by using Wilcoxon Signed Ranks Test and the data shown are by means of ± SD, *$p<0.05$.

As it is seen from table 5, the serum zinc levels and PMN's phagocytic activity of healthy young volunteers significantly increased after zinc supplementation ($p<0.05$).

On the other hand, there was no significant difference between the PMN's intracellular killing activity of healthy young volunteers before and after zinc supplementation ($p>0.05$).

DISCUSSION

Zinc is a cofactor of over 300 metalloenzymes, which cannot function in its absence. Thus zinc exerts control in the expression of many mediator factors and regulatory molecules of the immune response. There is extensive evidence for the effect of zinc deficiency on leukocyte functions. The deleterious effect of zinc deficiency in lymphocytes and granulocytes was pointed out before. The presence of zinc in plasmic membrane contributes to its stabilization and integrity by activating the cytoskelatal level. This might explain the depression of phagocytosis, oxygen consumption and bactericidal activity induced by zinc in phagocytic cells (9,10). Serum zinc level decreases when the age increases (8). We have found that the mean serum zinc level in older hypertensive patients was significantly lower than that of healthy young volunteers (Table 3). The underlying diseases of those patients, the hypertensive drug (enalapril maleate) used for their treatment and aging may be the cause of that low zinc level.

In this study we found that PMN's phagocytic activity in 4 of the 10 older hypertensive patients with serum zinc levels between 0.49-1.06 ppm was low. On the other hand the intracellular killing activity of the same patients was found to be low in 5 of those 10 patients. Additionally both PMN functions of the 10 healthy young volunteers were normal.The serum zinc levels of older hypertensive patients were lower than the serum zinc levels of the healthy

young volunteers. Thus we can attribute the decreased PMN functions to their low serum zinc levels.

It has been reported that patients with this underlying disease had low serum zinc levels (16,17). These patients have been using enalapril maleate in their treatments. This drug may also alter their serum zinc level. It has been shown that hypertensive patients on enalapril maleate treatment might have zinc deficiency (18,19). All of the older hypertensive patients in our study were hypertensive and have been using these drugs as part of their treatment.

The underlying chronic disease, as well as aging, impair the immune functions in older hypertensive patients. Moderate or undetectable zinc deficiency may contribute to this impairment. Non-hospitalized older hypertensive patients with significant clinical symptoms of zinc deficiency were previously noted [9]. It has been shown that even mild zinc deficiency could reduce the production and the activity of cytokines such as IL-1,2,3,4,5,6, INF-α, INF-γ and TNF-α. Mild zinc deficiency may also be accompanied by an imbalance in Th1 and Th2 cell functions resulting in impaired resistance to infections (9,10) .

Zinc therapy improves lymphocyte functions and viability in chronic uremic patients in which the abnormal zinc metabolism may play a role in the impairment of cellular immunity (9). Zinc supplementation has been shown to improve the resistance of older hypertensive people to infections by increasing their immunity. Marginal zinc deficiency and suboptimal zinc status have been observed in several "at risk" population groups such as older hypertensive people.Administration of adequate zinc supplementation could prevent the impairment of immune system and substantially improve the host resistance to infections in these populations (10).

The serum zinc levels of older hypertensive patients increased after zinc supplementation, but their phagocytic activity did not significantly increase. The older hypertensive patients were treated with enalapril maleate during their theapy. It was shown that enalapril significantly reduced human PMNs' phagocytic activity (20) .

Bogden, et al (21) investigated the effects of zinc supplementation (15 and 100 mg/day versus placebo for 3 months) on 103 healthy older hypertensive people. They found that plasma zinc levels significantly increased after l00mg daily zinc intake.

In the present investigation possibly the dose (22mg zinc picolinate/day/oral) given to the older hypertensive patients and period of zinc supplementation (30 days) were inadequate . When kept in mind that these hypertensive patients were receiving enalapril maleate during their therapy, it is predictible that the proper dosage and period of zinc supplementation may increase their PMN's the phagocytic and intracellular killing activities.

We conclude that PMN functions (phagocytosis and intracellular killing activity) were decreased in some of the older hypertensive patients. In our opinion, since enalapril maleate and the other hypertensive drugs have deleterious effects on serum zinc levels of older hypertensive patients, zinc supplementation might be beneficial. Adequate zinc supplementation may improve PMN functions both in older hypertensive patients and healthy young volunteers.

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