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Effects of oral zinc sulfate applications at different pH (ascorbic acid, vinegar of grapes and distillated water) on serum zinc levels in rabbits*

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Summary: The oral zinc (Zn) applications may be required in the case of Zn losses in the body. Lots of Zn forms and compounds are used for this aim. Sulfate form of Zn is used in a widespread manner with in distillated water or other diluent solutions. The purpose of this study is to reveal the effect of some acidifier agents as diluent solutions (therapeutic dose of ascorbic acid in distillated water and pure vinegar of grapes) versus distillated water, well known and commonly used. Thirty New Zealand rabbits in three equal groups were used in this study. Zinc sulfate solutions with different diluents as above were given to groups orally. Before and after (2,5 hours later) oral applications, blood samples were collected via cardiac puncture under general diethyl ether anesthesia. Atomic absorption spectrometer equipped with flame system was employed. Significant differences ($p \leq 0.001$) were found between first and last serum Zn levels in all groups. There were also significant differences among last samples of groups (minimum $p<0.05$). Throughout the experiment time significant differences were found between control group and acidifier groups (ascorbic acid and vinegar of grapes) ($p<0.05$), but not between two acidifier groups ($p>0.05$)**.** Significant correlation was detected between pH and serum Zn levels of oral solutions ($r = -0.838$, $p<0.001$). It was also observed that the oral solutions pH of $ZnSO_4$ influenced the serum Zn level significantly (r^2: 70.1%, $p<0.001$).

Consequently, the acidity of oral $ZnSO_4$ fortification solution affected the serum Zn level. While the pH level of oral $ZnSO_4$ solutions was decreasing, serum Zn level increased. The therapeutic dose of ascorbic acid in distillated water was more effective than pure vinegar of grapes

Key words: Ascorbic Acid, serum Zn level, rabbit, vinegar of grapes, Zn fortification

Tavşanlarda farklı pH'da (askorbik asit, üzüm sirkesi ve saf suya) oral çinko sülfat uygulamalarının serum çinko düzeyleri üzerine etkileri

Özet: Vücutta çinko (Zn) kaybının olması durumunda oral Zn uygulamalarına gerek duyulabilir. Bu amaçla çok sayıda Zn formu veya bileşiği kullanılmaktadır. Zn'nin sülfat formu geniş çapta safsu veya diğer sulandırma solüsyonları ile birlikte yaygın bir şekilde kullanılmaktadır. Bu çalışmanın amacı, sulandırma solüsyonları halinde bazı asitleştirici ajanların (saf su içinde tedavi dozunda askorbik asit ve saf üzüm sirkesi) iyi bilinen ve yaygın bir şekilde kullanılan saf suya karşı etkisinin ortaya konmasıdır. Bu çalışmada üç eşit grup halinde 30 Yeni Zelanda ırkı tavşan kullanıldı. Yukarıda bahsedildiği gibi farklı sulandırmalar ile $ZnSO_4$ gruplara oral yolla verildi. Oral uygulama öncesi ve 2,5 saat sonrası dietil eter genel anestezisi altında kalpten kan örnekleri alındı. Alev sistemli atomik absorbsiyon spektrofotometre kullanıldı. Tüm gruplarda ilk ve son serum Zn düzeyleri arasındaki fark önemli bulundu ($p \leq 0.001$). Grupların son örneklerinin arasında da önemli farklılıklar vardı (en az $p<0.05$). Deney süresi boyunca kontrol grubu ve asit grupları (askorbik asit ve üzüm sirkesi) arasında önemli farklılık bulundu ($p<0.05$), fakat iki asit grubu arasında fark gözlenmedi ($p>0.05$). Oral solüsyon pH'ı ve serum Zn düzeyi arasında önemli korelasyon tespit edildi ($r = -0.838$, $p<0.001$). Aynı zamanda $ZnSO_4$ oral solüsyon pH'ının serum Zn düzeyini önemli ölçüde etkilediği gözlendi (r^2: 70.1%, $p<0.001$).

Sonuç olarak, oral $ZnSO_4$ takviyesinin asiditesi serum Zn düzeyini etkiledi. Oral $ZnSO_4$ solüsyonunun pH'ı azalırken, serum Zn düzeyi arttı. Distile su içinde askorbik asitin terapotik dozu saf üzüm sirkesinden daha etkili bulundu.

Anahtar sözcükler: Askorbik asit, serum Zn düzeyi, tavşan, üzüm sirkesi, Zn takviyesi.

Introduction

Zinc (Zn) is an essential mineral and contributes several body protein and enzyme (3, 38). So a number of biochemical processes need Zn (16,25) and Zn deficiency causes numerous metabolic or functional disorders. Some of them are carbohydrate, fat and protein metabolism disorders and immune system dysfunctions (11,21,32).

There are numerously reports on stress maker cases which is cause Zn deficiency and low serum Zn level. Karademir (13) reported that stress induced by food and

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mouth diseases vaccination caused decrease of serum Zn level. Leblondel et al. (18) observed that thyroparathyroidectomy caused low serum Zn level. As well as diarrheal diseases, pneumonia, seasonal stress, serum Zn levels decrease (35) and deficiency sings develop (13,22). Low Zn levels raise risk and severity of common infections and importance of zinc for newborn's growth (11). Additionally Zn deficiency cause depressed immune function, neurological abnormalities, impaired growth and development, increased susceptibility to infection and infection severity etc (28,29).

Like these cases some drugs included different Zn compounds are used for the fortification of Zn reserves of the body. A great deal chemical compounds of Zn have been proposed for the Zn supplementation. However zinc sulfate ($ZnSO_4$) is commonly used as it has low cost, high effectiveness and solubility (2,8,10). $ZnSO_4$ used for treatment or preventive medicine as alone or in combination such as ascorbic acid (24).

Zinc is absorbed principally throughout the small intestine of the animals. The greatest absorption site of the small intestine is reported as duodenum (6,20). However, previously this side was known as large intestine (7). The excess or inadequate amounts of minerals must always be considered. Because interaction among minerals is well known and the excessive amount of Zn in diet or organism can affect badly the other minerals levels, as well as low level of Zn (4,19,23).

It's known that approximately dietary Zn is absorbed in the proportion of 10% (20,25). Application way, type of Zn compounds, requirements of the organism, also the level of other minerals (Ca, Cu, Fe etc) closely affect the intestinal Zn absorption (7,9,17,29). There are some investigations which is express that Zn absorption can be involved in gastro intestinal and food pH level. Yamagucci et al. (37) reported that a single oral administration of zinc significantly increased the acidity of gastric contents. It was reported that absorbability of zinc sulfate at low pH was high than neutral pH (28). Grace and Lee (6) reported that acid nature of abomasum ensure that adequate amounts of Zn are available for absorption from small intestine. Similarly, Handerson et al. (8) found that Zn at low gastric pH ambient was absorbed more than other pH ambient groups. Contrarily, it was reported that low gastric pH may not be a prerequisite for normal intestinal Zn absorption from food (36).

Generally distillated water is used widespread in drug industry to dissolve of $ZnSO_4$. Oral therapeutic dose of Zn (as sulfate) was reported as 0,5 mg/kg and this dose reaches in 2-3 hours to maximum plasma level (9,17,28,30). The ascorbic acid is also combined for the fortification of therapy (24) and has acidifier effect for dilution solutions (27). Vine grape is a low pH food additive which is widely used in food preparations. There is also no information about ascorbic acid and vinegar of grapes as acidifier agents on serum Zn levels with oral $ZnSO_4$ applications.

Whence, the aim of this study was to investigate effects of oral $ZnSO_4$ applications with acidifier diluents as ascorbic acid and vinegar of grapes with compare of distillated water on serum Zn levels.

Materials and Methods

Animals, procedures and blood collections: The study was approved by the Ethics Committee of University of Kafkas (Approval No. 2009-14-09). Clinically healthy 30 male, New Zealand rabbits, aged between 6 and 8 months and weighing 2720 ± 131 g were used in this study. The animals divided into three equal experimental groups. Administrations of treatment groups were as follows; A: Zinc Sulfate + distillated water, B: Zinc Sulfate + Ascorbic acid and C: Zinc Sulfate + grape-vinegar.

The oral solutions included 0.5 mg Zn as Zinc Sulfate heptahydrate (Fluka, 96500) in one milliliter for all groups. The $ZnSO_4$ dissolved in distillated water for group A, in distillated water with 16,5 mg ascorbic acid (Fluka, 95209) for group B and in absolute vinegar of grapes (Moody international certification, ISO 22000-2005) for groups C.

No food was given to experimental groups overnight. The rabbits were weighed and two milliliters of the blood was collected via cardiac puncture under the ether anesthesia. After the blood collection, $ZnSO_4$ solutions were applied orally at the dose of one milliliter per kg body weight. The time for the maximum plasma level of oral $ZnSO_4$ with distillated water reported as between two to three hours (10,14,17,28,30). Therefore, second blood collection time determined as 2 hours and 30 minute later oral $ZnSO_4$ applications.

The animals were fed by a commercial animal food, hay and tap water. The food, hay and water were given *ad libitum* before and during experiment. Zn determinations were performed by Atomic Absorption Spectrometer equipped with flame system (FAAS) (Thermo Elemental S4, Thermo Electron Corporation, Cambridge, UK). The analyses results of diet Zn levels were as follows; food: 26.53 mg/kg in dry matter, hay: 21.62 mg/kg in dry matter and water: 0.071 mg/L.

The commercial food (ISO 9001:2000, ISO 22000:2005) was used. The dry matter, crude protein, crude fibre, crude ash, acid insoluble ash, acid insoluble ash and metabolizable energy were analyzed with Diode Array 7200 (DA 7200) device having periodic maintenance and calibrations. All the food composition data were reported by manufacturer (Table 1).

Table 1: Composition of food given to rabbit
Tablo 1: Tavşanlara verilen yemin kompozisyonu

Diet composition	
Dry matter (%)	88
Crude protein (%)	17
Crude fibre (%)	12
Crude ash (%)	10
Acid insoluble ash (%)	1
Calcium (%)*	1,5
Phosphorus (%)*	0.75
NaCl (%)*	0.6
Vitamin A (IU/kg)*	5000
Vitamin D_3 (IU/kg)*	600
Vitamin E (mg/kg)*	25
Metabolizable energy (kcal/kg)	2600

Raw-materials for this composition: Barly, corn, corn chaff, corn glutein, wheat, rye, wheat-bran, cottonseed meal, sunflower meal, dicalcium phosphate and vitamin, mineral premix.
* Calculated

Laboratory analyses: Orion 4-Star Portable pH/lSE Meter (96-09) equiped with Triode, gel-filled epoxy-body pH electrode (Orion 9107BNMD) was used for analyses of solutions' pH. Calibration of the device was made with commercial Orion certified standard solutions for pH 4, 7 and 10 (Orion 910410, 910710 and 911010 respectively) purchased from Thermo Electron Corporation Beverly, USA.

Serum zinc measurements were made by FAAS. After the coagulation of blood, blood serum was separated with centrifuge (15 minute at 3500 revolutions per minute-rpm). De-proteinisation of serum was made with Trichloroacetic acid (TCA) (20% w/v) (Merck 100810). One ml of serum was mixed and heated with TCA for 15 minute in 80 ºC and mixture was centrifuged. Supernatant used for FAAS measurements (12). Standard solutions for Zn supplied from Fluka Chemie GmbH, Switzerland (Fluka 96457).

Accuracy control of FAAS was performed using previously known standard solutions for Zn measurements. This standard solution was aspirated for 6 times per 10 samples during analyses and mineral levels were measured. Coefficients of variations (CV) for this parameter was calculated from this obtained findings. CV was found to be 4.07% (12). All lab-ware used were made of PTFE material.

Statistical analysis: Statistical analyses were performed using SPSS statistical software version 10.0.1 (33). Data were presented as means ± S.E.M.

One-Way ANOVA was used for comparison of first and last serum samples findings and was also used for comparison of groups (5). Differences of Zn levels throughout the experimental period as well as interaction between time and groups were analyzed by repeated measurement ANOVA (RM ANOVA) (14,15). Duncan test was employed for multiple comparisons. Pearson correlation test was used to determine the relationship between solution pH and last serum Zn levels. Linear regression analysis was used to observe the effect of solution pH on serum Zn levels.

Results

The pH levels of oral $ZnSO_4$ solutions and also mean values of serum Zn levels were summarized in Table 2. Serum Zn levels increased after 2 hours and 30 minutes later of oral $ZnSO_4$ applications ($p<0.001$). The differences between group B and C (acidifier groups) were significant ($p<0.05$). However, the statistical difference between acidifier groups and group A were higher ($p<0.001$) than between groups B and C. The increase of the serum Zn levels on last day were correlated to pH of oral $ZnSO_4$ solutions ($r = - 0.838$, $p<0.001$) (Figure 1). It was also observed that the effect of solutions pH on serum Zn level were significant with the regression equation of Zn µg/dl = 343 – 33.4 pH, r^2 = 70.1 %, $p<0.001$.

Table 2: First and last serum Zn levels according to solution groups
Tablo 2: Solüsyon gruplarına göre ilk ve son serum Zn düzeyleri

Groups	Oral $ZnSO_4$ solution pH of the groups	Serum Zn levels (µg/dl)	
		First Samples	Last Samples
A	5.17	129.20 ± 5.99^{B}	171.79 ± 8.82^{Ac}
B	2.37	129.43 ± 7.09^{B}	272.04 ± 11.4^{Aa}
C	2.71	125.32 ± 7.64^{B}	243.86 ± 4.82^{Ab}

^{A,B}: Means with different superscript letters are significantly different in lines ($p<0.001$).
^{a,b,c}: Means with different superscript letters are significantly different in columns ($p<0.05$).
Description of groups: A: Zinc Sulfate + distillated water, B: Zinc Sulfate + Ascorbic acid and C: Zinc Sulfate + grape-vinegar.

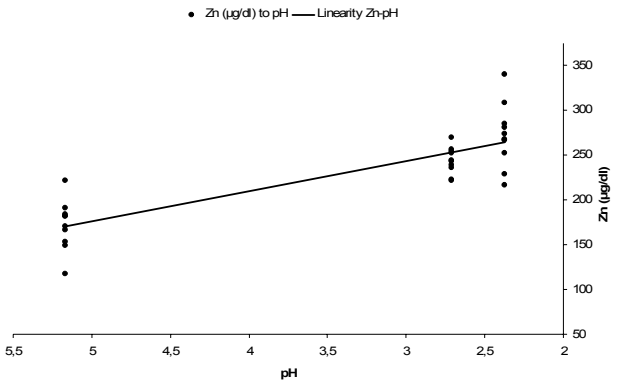

Figure 1: Statistically significant negative correlation between solutions' pH and last serum Zn concentration ($r = - 0.838$; $p<0.001$) with the Zn µg/dl = 343 – 33.4 pH regression equation (r^2:%70.1, $p<0.001$).
Şekil 1: Solüsyonların pH'ı ve serum Zn konsantrasyonları arasında istatistiken önemli negative korelasyon ($r = - 0.838$; $p<0.001$). Regresyon formülü: Zn µg/dl = 343 – 33.4 pH, r^2: 70.1%, $p<0.001$.

Throughout the experiment time, RM ANOVA test results showed that there were significant difference within time ($p<0.001$). There were also significant interactions between time and serum Zn levels of groups ($p<0.001$). Zn level in group A was less than in group B and C in last samples of groups ($p<0.05$). However, Zn levels were not different between group B and C ($p>0.05$) (Figure 2).

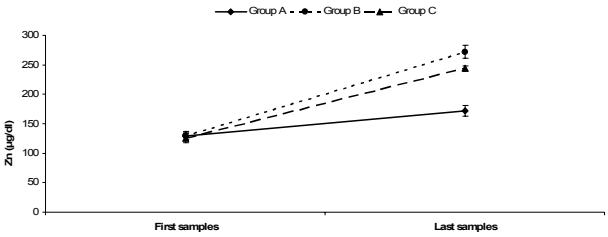

Figure 2: Zn levels of the groups during the experimental period.
Şekil 2: Deneysel periyot süresince grupların Zn düzeyleri.

Discussion and Conclusion

Serum Zn level can decrease during some health disorders (13-15). Fortification of oral $ZnSO_4$ may play an important role in achieving adequate serum Zn level (1,10,28,35).

In group A, dissolving solution for oral $ZnSO_4$ applications was only distillated water and this group was considered as control group. The other two groups received $ZnSO_4$ with acidifier dissolving solutions. Ascorbic acid requirements during infections or other diseases are increase and require additional prophylactic supplementation (26). The therapeutic dose of ascorbic acid is reported as 16.5 g/kg (30,31). It is also known that ascorbic acid and $ZnSO_4$ can use together in practice of human drug industry in a widespread manner (24). Ascorbic acid is reported as acid nature chemicals and the pH level of one mole ascorbic acid in water reported as 1.0-2.5 (27). The solution pH level of $ZnSO_4$ with ascorbic acid was 2.37 in group B of this study predictably. Grape vinegar is well known a food additive with acidic nature. Pure vinegar of grapes was used for preparation of oral $ZnSO_4$ solution of group C. In this study, the pH level measured as 2.71 for $ZnSO_4$ solution of group C.

Some literatures stated that intestinal Zn absorption could be affected by additives or other manipulations (18,34). Absorbability of the different Zn salts presumably depends on their solubility in aqueous solution (28). The relative bioavailability of different Zn compounds that may be used in food fortification may be different (10). The bioavailability of orally administrated Zn as a drug seems to be also dependent on the amount and the nature of the food with which it is administered (17). Oral $ZnSO_4$ solutions with various pH levels were applied to the rabbits in this study. For the regulation of solutions' pH distillated water, ascorbic acid at therapeutic dose in distillated water and absolute grape-vinegar were used. Serum Zn levels for all groups increased after the oral $ZnSO_4$ applications as expected. It's well known that the most important factor affecting absorption is the Zn content of diet. However Zn-deficient animals absorb a higher percentage of administrated Zn. Net absorption of intestinal Zn was reported as high as 80% in Zn-deficient calves. Some times a reduction of less than 10% can be occurring with high-Zn diet (20,25). Throughout the experimental time increase of serum Zn levels were observed in all groups. However, this increase in acidifier groups (B and C) were higher than group A ($p<0.05$). There are conflictive reports about Zn absorption related to pH of food, intestinal or gastric ambient (6,8,28,36). It was reported that absorbability of zinc sulfate at low pH was high than neutral pH (28). Grace and Lee (6) reported that acid nature of abomasum ensure that adequate amounts of Zn are available for absorption from small intestine. Similarly, Handerson et al. (8) found that Zn at low gastric pH ambient was absorbed more than other pH ambient groups. Contrarily, it was reported that low gastric pH may not be a prerequisite for normal intestinal Zn absorption from food (36). In this study RM ANOVA findings clearly showed that the acidifier agents affected to increase of serum Zn level during experiment. Comparison results of last day findings by means of One-Way ANOVA supported the RM ANOVA results and indicated that there was a significant difference between group B and C ($p<0.05$) while there were significant different between acidifier groups and group A ($p<0.001$). The correlation between solution pH and Zn level of last serum sample was found significant ($r = -838$, $p<0.001$), and the pH of solutions were found highly effective on serum Zn increase according to regression analyses ($r^2 = 70.1p<0.001$). Findings of this study supported to most of the investigations (6,8,28) but not the finding of Turnbull et al (36).

All data clearly showed that diluent solutions for oral $ZnSO_4$ prepared with acidifier agents as ascorbic acid and vinegar of grapes were effective versus distillated water according to serum Zn level increase. Besides, as acidifier diluent, the therapeutic dose of ascorbic acid in distillated water was more effective than pure vinegar of grapes. Thus, it was observed that solution pH of oral $ZnSO_4$ applications was found significantly effective on serum Zn levels with negative correlation.

Consequently, it was observed that Zn levels were affected by the oral solutions pH. Serum Zn levels were increased with the increased level of acidity. Ascorbic acid at therapeutic doses was more effective than pure vinegar of grapes.

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