The Effects of Taurine, Melatonin and N-Acetylcysteine on Cadmium Exposure Bone Changes “The Surprising Effect of Taurine”

Taurin, Melatonin ve N-Asetilsisteinin Kadmiyum Maruziyeti Sonrası Kemik Değişikliklerine Etkileri “Taurinin Şaşırtıcı Etkisi”

Nurettin Tastekin 1, Nurettin Aydoğdu 2, Gulya Durmus Altun 3, Hakan Erbas 4, Kaan Uzunca 1, Murat Birtane 1, Mustafa Kaplan 5 Murat Erem 6

1Trakya University, Faculty of Medicine, Department of Physical Medicine and Rehabilitation, Edirne, Turkey
2Trakya University, Faculty of Medicine, Department of Physiology, Edirne, Turkey
3Trakya University, Faculty of Medicine, Department of Nuclear Medicine, Edirne, Turkey
4Trakya University, Faculty of Medicine, Department of Biochemistry, Edirne, Turkey
5Trakya University, Faculty of Medicine, Department of Urology, Edirne, Turkey
6Trakya University, Faculty of Medicine, Department of Orthopaedic Surgery and Traumatology, Edirne, Turkey

ABSTRACT

Background: Chronic environmental and occupational exposure to cadmium can result in skeletal system changes. The main objective of the present study was to investigate and compare the effects of taurine, melatonin and N-acetyl cysteine on cadmium exposure induced bone density loss.

Methods: 90 adult male Sprague-Dawley rats were allocated into four main groups: Group I was the control group; Group II was the “cadmium exposure” group; Group 3 was “cadmium exposure for 3 months + concurrent antioxidant administration” group. The concept of Group 4 was cadmium exposure for 3 months + subsequent antioxidant administration. Bone mineral density values were evaluated in all the groups. Serum calcium, phosphorus, alkaline phosphatase (ALP) enzyme activities and 24 hours urine calcium excretion levels were measured. Kruskal–Wallis test was used to compare the all groups. Between two group comparisons, the Mann–Whitney U test was used.

Results: There was no significant difference in terms of bone mineral density values only between control group and cadmium exposure group (p>0.05). Mean bone mineral density values obtained in “cadmium + concurrent taurine” and “cadmium + subsequent taurine” groups were significantly lower than all the other groups (p<0.05). 24 hours urine calcium excretion levels were significantly higher in groups which taurine and n-acetylcysteine were administered after cadmium exposure.

Conclusion: Taurine, which is thought to have protective effects as an antioxidant caused a marked bone damage after exposure to cadmium. Further studies are needed to clarify this effect of taurine.

Key Words: Cadmium exposure, Taurine, N-acetylcysteine, Bone, Antioxidant, Melatonin

Received: 10.19.2018    Accepted: 01.31.2020

ÖZET

Amaç: Kronik olarak çevresel ve mesleki kadmiyum maruziyeti, iskelet sistemi üzerinde değişikliklere neden olur. Bu çalışmanın temel amacı ise taurin, melatonin ve N-asetil sisteinin, kadmiyum maruziyetinin neden olduğu kemik yoğunluk kaybı üzerindeki etiketlerini incelemek ve karşılaştırmaktır.

Yöntem: 90 adet yetişkin erkek Sprague-Dawley cinsi çıkan dört ana gruba ayrılmıştır. Birinci grubu kontrol grubu, ikinci grubu kadmiyum maruziyetinin olduğu grup, üçüncü grubu 3 ay kadmiyum maruziyet ve eş zamanlı antioksidan verilen grupdur. Dördüncü grubun özelliği ise 3 ay kadmiyum maruziyeti ve daha sonrasında antioksidan verilmişdir. Bu çalışmada serum kalsiyum, fosfor, alkalen fosfat (ALP) enzim aktivitesi ve 24 saat idrar kalsiyum atılım seviyeleri ölçülmüştür. Bir dört gruba Kruskal-Wallis testi ile karşılaştırmıştır. İki gruba karşılaştırma testleri ise Mann-Whitney U testi ile yapılmıştır.

Bulgular: Kemik mineral yoğunluklarının açısından kontrol grubu ile kesin maruziyeti olan grup arasında anlamlı fark yoktur (p>0.05). Kadmiyum ve eş zamanlı taurin verilen grup ile kemik maruziyet ve daha sonra taurin verilen gruplardan elde edilen ortalama kemik mineral yoğunlukları diğer bütün gruplardan düşüktür (p<0.05). Kadmiyum maruziyeti sonrasında taurin ve n-asetilsistein verilen gruplarda 24 saat idrar kalsiyum atılım seviyeleri belirgin olarak yüksektir.

Sonuç: Kadmiyum maruziyeti sonrası antioksidan olarak koruyucu etkiyi olduğu düşünülen taurin, belirgin kemik hasarı oluşturmakta. Taurinin bu etkisini netleştirmek ve açığa çıkarmak amacıyla daha fazla çalışmaya ihtiyaç vardır.

Anahtar Sözcüklere: Kadmiyum maruziyeti, Taurin, N-asetilsistein, Kemik, Antioksidan, Melatonin

Geliş Tarihi: 19.10.2018    Kabul Tarihi: 31.01.2020

ORCID IDs: NT 0000-0002-1033-806X, NA 0000-0002-6341-5137, GAD 0000-0002-9773-5390, HE 0000-0002-7261-4170, KU 0000-0002-0548-3748, MB 0000-0003-0294-4155, MK 0000-0002-6662-2051, ME 0000-0002-9743-5515

Address for Correspondence / Yazılma Adresi: Nurettin Tastekin, MD, Prof Fatih Mah. 40. Sok. Medibloklar 1. Kism D Blok No: 7, TR22030 Edirne-Türkey E-mail: zentastekin@yahoo.com

©Telêf Hakki 2020 Gazi Üniversitesi Tip Fakültesi - Makale metnine http://medicaljournal.gazi.edu.tr/web adresinden ulaşılabilir.
©Copyright 2020 by Gazi University Medical Faculty - Available on-line at web site http://medicaljournal.gazi.edu.tr/doi:http://dx.doi.org/10.12996/gmj.2020.129
INTRODUCTION

Cadmium, one of the non-essential, heavy metal elements is an important industrial, environmental polluter and known to have various toxic effects on living organisms (14). Chronic environmental and occupational exposure to cadmium can result in skeletal system changes, such as osteopenia, osteoporosis and osteomalacia, which all cause increased bone fragility. Sources of cadmium affecting human life are refined food, water pipes, coffee, tea, coal burning, shellfish, fertilizers and flue gases which occur during industrial production processes. In addition, smoking is an important source of cadmium (One cigarette contains 1-2 μg of cadmium) (10).

Osteoporosis is characterized by low bone density and micro-architectural deterioration of bone tissue. There is an increasing interest in environmental factors that may have potential to influence bone density. In this context heavy metals are one of the major agents accused. Substantial amount of heavy metals spread over the environment as a result of pollution due to continuous usage and misusage (3). Recent studies indicate that relatively low exposure concentration levels may also be associated with effects on the skeleton, such as low bone mineral density (BMD) although data are not consistent (4, 19,20). The negative effect of cadmium on bone has been supported by determined associations between urinary cadmium and increased risk of fractures (1). Both a direct effect on bone via increased bone resorption, and a secondary effect via the kidney damage are plausible mechanisms (17). Long-term exposure to low-dose cadmium has been associated with tubular impairment leading a loss of reabsorptive capacity and abnormal urinary excretion for calcium (12). Besides it has been reported that the inhibitory effects of cadmium on the activation of vitamin D in renal cortical cells can lead to decreased calcium absorption and negative calcium balance in cadmium-exposed rats (8). When the human organism is exposed to the cadmium, it becomes a powerful competitor of calcium in biochemical processes (24). This competition may play an important role in deterioration of the bone quality.

Cadmium-induced cellular and structural damages are reported to be associated with oxidative stress balance. Therefore, antioxidants should be considered as molecules having protective effect against tissue damages that may occur during the process [2]. Some of the antioxidant amino acids are known to be used as protectors against cadmium exposure. Especially, melatonin the main product of pineal gland is reported to catch free radicals in various tissues and organs and to have indirect antioxidant activity (20).

Taurine, which is emphasized as having an antioxidant property in studies, can be found freely in mammalian tissues. Taurine is an amino acid which is synthesized from cysteine and does not get into the protein structure (16). N-acetyl cysteine has been reported to have protective effect against tissue damage which happens due to free radicals (11). Taurine, melatonin and N-acetylcycteine have been shown to play protective roles against tissue damage in many experimental studies including cadmium usage as a tissue damage promoter (11,16,20).

The main objective of the present study was to investigate and compare the effect of taurine, melatonin and N-acetylcycteine on cadmium exposure bone.

METHODS

Animals

Ninety adult male Sprague-Dawley rats weighing 340–370 g were used. The animals were housed in groups of nine per cage. They were maintained under standard conditions (12/12 light–dark cycle, room temperature, 22±2°C) and fed with standard rat chow. The animals were obtained from the “Animal Facility of the Trakya University School of Medicine”.

Experimental groups

After the provision of the local ethics committee approval, 90 adult male Sprague-Dawley rats were allocated into four main groups. The distribution properties and the processes performed in the groups were as follows: Group 1 was the control group with no intervention; Group 2 was the “cadmium exposure” group and the rats in this group were exposed to cadmium for 3 months by drinking 200 mg/L CdCl2 (cadmium chloride) (Fluka, U.S.A.) added water; Group 3 was “cadmium exposure for 3 months + concurrent antioxidant administration” group. The Group 3 concept was shared by 3 groups according to the antioxidant administrated. Group 3a was Taurine group. (% 1 taurine (Sigma, U.S.A.), Group 3b was melatonin group (0.02 % melatonin (Sigma, USA) and finally Group 3c was N-acetylcycteine group (0.5 % N-acetylcycteine (Sigma, U.S.A.). The concept of Group 4 was cadmium exposure for 3 months + subsequent antioxidant administration which was shared again by 4 groups according to the antioxidant administrated. Group 4a was Taurine group (%4), Group 4b, Melatonin (%0.08) and Group 4c, N-acetylcycteine (%2), and finally Group 4d, the control group of this subsection with tape water administration. The details on group configurations can be seen in table 1.

| Table 1. The distribution of all groups |
|----------------------------------------|
| **EXPOSURE** | **DOSAGE** |
| Group I | n=10 | Drinking water |
| Group II | n=10 | 200 ppm CdCl2 |
| Group III | n=10 | a. 200 ppm CdCl2 | %1 taurine |
| | | b. 200 ppm CdCl2 | %0.02 melatonin |
| | | c. 200 ppm CdCl2 | %0.5 N-acetylcycteine |

**Protective groups**

| Group IV | n=9 | a. 200 ppm CdCl2 | %4 taurine |
| | | b. 200 ppm CdCl2 | %0.08 melatonin |

**Treatments groups**

| | n=10 | a. 200 ppm CdCl2 | %2 N-acetylcycteine |

The animals were housed individually in metabolic cages, to collect 24-h urine specimens for analyzing glomerular filtration rate (GFR). At the time of sacrifice, the rats were anesthetized with 10 mg/kg of xylazine (Rompun®, Bayer, Turkey) and 50 mg/kg of ketamine (Ketalar®, Eczacibasi, Turkey) (Sacrification time was planned after the protocol was completed in all rats). Blood samples were collected by cardiac puncture. The femur bones of the specimens were extracted under anesthesia. The rest of the bone tissue was stored at ~80°C. Blood samples were centrifuged (1,500 g for 10 min at 4°C) immediately, and plasma samples were stored at ~80°C until assayed.

As one rat from each of Group 4a and 4b died during the study period, evaluations in these groups were performed on the rest of the rats.

Biochemicals assay

Serum calcium, phosphorus, alkaline phosphatase (ALP) enzyme activities and 24 hours urine calcium levels were measured spectrophotometrically using a UniCel Dxc 800 Synchron Clinical System (Beckman Coulter, USA) in the Central Laboratory Unit.

Bone densitometry

DEXA scans were performed with a fan beam QDR 4500 densitometer (Hologic, Inc., Bedford, MA) calibrated daily in accordance with the manufacturer’s recommendations. The regional high-resolution mode of the small animal scan protocol (scan field 5.0 [width] x 6.0 [height] cm2, pixel size 0.31 mm2, scan time 2 min) was used. The coefficient of variation (CV) for the measurements (determined by ten separate scans in two control rat after repositioning) was <2%.
All densitometric parameters were analyzed by the same examiner, and measurements in control and CdCl2 exposed rats after respective periods were made on the same day.

**Ethics**

All experimental protocols were approved by the “Trakya University School of Medicine Animal Care Ethics Committee”.

**Statistical analysis**

The results were expressed as mean ± S.D. Kruskal–Wallis test was used to compare all the groups. In two group comparisons, the Mann–Whitney U test was used. P values below 0.05 were considered to be statistically significant.

**Table 2.** The mean bone mineral density values of the groups

| GROUP | BMD (g/cm²) |
|-------|-------------|
| I     | 0.1679 ± 0.0093 |
| II    | 0.1654 ± 0.0081 |
| IIIa  | 0.1561 ± 0.0019* |
| IIIb  | 0.1671 ± 0.0010 |
| IIIc  | 0.1629 ± 0.0065 |
| IVa   | 0.1563 ± 0.0097* |
| IVb   | 0.1665 ± 0.0015 |
| IVc   | 0.1663 ± 0.0014 |
| IVd   | 0.1707 ± 0.0010 |
| Average | 0.1644 ± 0.0011 |

*p<0.05

Group 1 was the control group; Group 2 was the cadmium exposure group, Group 3 was cadmium exposure for 3 months + concurrent antioxidant administration group (a. Taurine, b. Melatonin, c. N-acetylcysteine), the concept of Group 4 was cadmium exposure for 3 months + subsequent antioxidant administration (a. Taurine, b. Melatonin, c. N-acetylcysteine, d. Drinking water)

**Table 3.** The biochemical values of all groups

| GROUP | Calcium values | Phosphorus values | Alkaline phosphatase | 24 hours urine calcium excretion |
|-------|---------------|------------------|---------------------|-------------------------------|
| I     | 9.16±0.77     | 5.14±0.81        | 83.20±17.83         | 3.82±2.02                     |
| II    | 8.98±0.45     | 5.74±0.64        | 87.40±36.41         | 6.98±2.09                     |
| IIIa  | 8.95±0.66     | 5.54±1.02        | 81.10±22.84         | 6.24±1.53                     |
| IIIb  | 9.01±0.87     | 5.45±0.99        | 69.20±20.90         | 6.63±1.15                     |
| IIIc  | 9.03±0.45     | 5.72±0.88        | 97.20±33.40         | 7.07±2.68                     |
| IVa   | 8.81±0.50     | 5.72±0.41        | 105.56±35.37        | 18.39±4.27*                   |
| IVb   | 8.83±0.45     | 6.03±0.71        | 87.50±26.16         | 4.72±0.88                     |
| IVc   | 8.48±0.35     | 5.56±0.58        | 98.90±33.75         | 11.62±4.79*                   |
| IVd   | 8.83±0.79     | 5.87±0.80        | 106.90±49.04        | 5.39±1.64                     |
| Average | 8.90±0.62   | 5.63±0.79        | 90.67±32.73         | 7.82±4.90                     |

*p<0.05

Group 1 was the control group; Group 2 was the cadmium exposure group, Group 3 was cadmium exposure for 3 months + concurrent antioxidant administration group (a. Taurine, b. Melatonin, c. N-acetylcysteine), the concept of Group 4 was cadmium exposure for 3 months + subsequent antioxidant administration (a. Taurine, b. Melatonin, c. N-acetylcysteine, d. Drinking water)

**RESULTS**

The study hypothesis anticipating that cadmium could have detrimental effects on bone mineral density (BMD) has not been proved clearly. There was no significant difference observed in comparison between Group I and II (p>0.05). Nevertheless the BMD values in the group which patients use protective taurine with cadmium (Group IIIa) and use taurine after cadmium exposure as a treatment agent (Group IVa) were significantly lower when compared with all other groups (p<0.05). Mean BMD values of all the groups are shown in table 2.

**DISCUSSION**

There are many studies which emphasized the bone damage and consequent bone loss effects of chronic cadmium exposure (4,14,19). The main purpose of this study was to investigate the probable protective effects of some antioxidant molecules such as taurine, melatonin and N-acetylcysteine against the bone damage provided by three months cadmium exposure. However, the cadmium exposure in the mentioned dosage did not reveal significant differences on bone mineral density and did not generate expected damage in bone tissue. On the other hand we have met a striking finding during general evaluation of the findings. Our basic hypothesis that taurine would show a positive effect on bone damage which occur due to oxidative stress collapsed and we observed significantly lower bone mineral density in groups taking taurine supplementation. Consistent to this finding, calcium excretion increased in taurine administered group.

Antioxidant supplementation during cadmium exposure has been reported to cause positive improvement by preventing lipid peroxidation. Taurine is thought to be an effective agent for preventing the oxidative stress related bone damage. Taurine has important roles in bone metabolism, and the taurine transporter which is a specific transport system, is shown to be expressed in osteoblasts. Taurine has been reported to inhibit experimental bone resorption and the formation and survival of osteoclasts as well. Fernandez-Perez et al. (17) has shown that striatal concentration on taurine decreases during cadmium exposure. Sinha et al. (15,21,22), in their various studies, has reported the preventing effect of taurine against hepatic oxidation, renal damage and oxidative stress in erythrocyte which may happen after cadmium exposure. Moreover, Choi (5,6) has claimed that there is no marked effect of taurine on BMD. In our study, there are question marks remaining about the negative effects of taurine. It is not clear this unexpected effect is due whether to direct dosage effect or true direct effect of cadmium.
As there is a consensus on the validity of dosage-time hypothesis about the effect of taurine on bone tissue, we think that the BMD lowering effect observed in this study may be due to the low dose of taurine administered and to the increasing effect of taurine on calcium excretion. New studies investigating the effects of taurine when used with cadmium and of the consequences of various dosages are needed.

Kaplan et al. (13) has shown the favorable effects N-acetylcysteine against the oxidative damage to the kidneys observed due to cadmium exposure. Doi et al. (7) reported that the N-acetylcysteine, as pretreatment before cadmium exposure, could have a protective role. Patrick (18) expressed the opinion that amino acids may have positive impacts against toxic effects of cadmium. Besides there are other studies revealing favorable effects of N-acetylcysteine by effecting the calcium excretion from the kidneys. We could not find a marked effect of N-acetylcysteine concurrently used with cadmium, on the amount of urinary calcium excretion. However although there was no effect of N-acetylcysteine on BMD when used after the cadmium exposure, 24 hours urinary calcium excretion increased in this model. Various models are needed for a detailed analysis on this effect.

There are some limitations in this study. One of the most basic limitations is that the cadmium exposure could not form bone damage. In some studies, deterioration in bone metabolism have been observed with low cadmium dose and in some of them related to the exposure time. Three months of exposure to cadmium exposure to bone damage models were planned. However, in our study there was no damage due to exposure. (23). Brzoska and Monisz-Jakonik (4) searched the bone damage using 1, 5 and 50 mg/L Cadmium for 3, 6, 9 and 12 months and observed significant BMD variations in bone tissue even with the lowest dosage administered during 6 months. What we experienced in this study was that it could not be possible to develop bone damage in dosages applied in classic trial programs. Thus we think longer application periods would be more efficient to construct this model.

Another question is that on what occasions can taurine cause bone damage, with simultaneous application added to cadmium or after exposure or when applied alone? The answer will clarify the direction of taurine effect when applied with cadmium. New models are being arranged and will be put into practice after the termination of this study.

We can conclude in the light of all analysis that taurine have generated unfavorable effects when used together with cadmium and thereafter. Efficiently planned studies are needed to answer the question whether there is a bone damage effect of taurine supplementation during cadmium exposure or not.

Conflict of interest
No conflict of interest was declared by the authors.

REFERENCES

1. Åkesson A., Bjellerup P., Lundh T, Lidfeldt J et al.: Cadmium-induced effects on bone in a population-based study of women. Environ Health Perspect, 2006; 114: 830–4.
2. Aydoğdu N, Kanter M, Erbaş H, Kaymak K: The effects of taurine, melatonin and acetylcysteine on nitric oxide, lipid peroxidation and some antioxidants in cadmium induced liver injury. Erciyes Med Journal 2007; 29: 89-96.
3. Baldwin DR, Marshall WJ: Heavy metal poisoning and its laboratory investigation. Ann Clin Biochem, 1999; 36: 267-300.
4. Brzoska MM, Moniszko-Jakonik J: Low-level lifetime exposure to cadmium decreases skeletal mineralization and enhances bone loss in aged rats. Bone, 2004; 35: 1180-91.
5. Choi MJ: Effects of taurine supplementation on bone mineral density in ovariectomized rats fed calcium deficient diet. Nutr Res Pract, 2009; 3: 108-13.
6. Choi MJ, Di Marco NM: The effects of dietary taurine supplementation on bone mineral density in ovariectomized rats. Adv Exp Med Biol, 2009; 643: 341-9.
7. Doi T, Purî P, Bannigan J, Thompson J: Pre-treatment with N-acetylcysteine upregulates superoxide dismutase 2 and catalase genes in cadmium-induced oxidative stress in the chick omphalocoele model. Pediatr Surg Int, 2011; 27: 131-6.
8. Feldman SL, Cousins RG: Influence of cadmium on the metabolism of 25-hydroxycholecalciferol in chicks. Nutr Rep Int, 1973; 8: 251-9.
9. Fernández-Pérez B, Caride A, Cabaleiro T, Lafuente A: Cadmium effects on 24h changes in glutamate, aspartate, glutamine, GABA and taurine content of rat striatum. J Trace Elem Med Biol, 2010; 24: 212-8.
10. Goyer RA: Toxic effects of metals. In: Klaassen, C.D. (Ed.), Casarett & Doull’s Toxicology: The Basic Science of Poisons, fifth ed. McGraw-Hill, New York, 1996.
11. Hashimoto K, Pinkas G, Evans L, Liu H, Al-Hasan Y, Thompson LP: Protective effect of N-acetylcysteine on liver damage during chronic intrauterine hypoxia in fetal guinea pig. Reprod Sci, 2012; 19: 1001-9.
12. International Programme on Chemical Safety. Cadmium-Environmental Health Criteria 134. Geneva: World Health Organization. Available http://www.inchem.org/documents/ehc/ehc/ehc134.htm.
13. Kaplan M, Atakan IH, Aydoğdu N, Aktöz T, et al: Influence of N-acetylcysteine on renal toxicity of cadmium in rats. Pediatr Nephrol, 2008; 23: 233-41.
14. Karabulut-Bulan Ö, Koyutürk M, Bolkent F. et al: Siçan tiroid bezinde kadmium hasanına karşı C vitaminii, E vitaminii ve selenyumun kombine kullanımının etkileri. Cerrahpaşa Tip Dergisi 2004; 35: 174-80.
15. Manna P, Sinha M, Sil PC: Taurine plays a beneficial role against cadmium-induced oxidative renal dysfunction. Amino Acids, 2009; 36: 417-28.
16. Nakamura T, Ogasawara M, Koyama I, Nemoto M, et al: The protective effect of taurine on the biomembrane against damage produced by oxygen radicals. Biol Pharm Bull 1993;16: 970-2.
17. Nordberg M, Jin T, Nordberg GF: Cadmium, metallothionein and renal tubular toxicity. IARC Sci. Publ 1992; 118: 293-7.
18. Patrick L. Toxic metals and antioxidants: Part II. The role of antioxidants in arsenic and cadmium toxicity. Altern Med Rev, 2003; 8:106-28.
19. Rignelli-Hydbom A, Skerfving S, Lundh T, Lindh CH et al: Exposure to cadmium and persistent organochlorine pollutants and its association with bone mineral density and markers of bone metabolism in postmenopausal women. Environ Res 2009;109: 991-6.
20. Reiter RJ, Tan DX, Burkhardt S: Reactive oxygen and nitrogen species and cellular and organismal decline: amelioration with melatonin. Mech Ageing Dev, 2002; 123: 1007-19.
21. Sinha M, Manna P, Sil PC: Induction of necrosis in cadmium-treated hepatic oxidative stress and its prevention by the prophylactic properties of taurine. J Trace Elem Med Biol, 2009; 23:300-13.
22. Sinha M, Manna P, Sil PC: Taurine protects the antioxidant defense system in the erythrocytes of cadmium treated mice. BMB Rep, 2008; 41:657-63.
23. Tang L, Chen X, Bao Y, Xu W, Lv Y, Wang Z, Wen X. CT Imaging Biomarkers of Bone Damage Induced by Environmental Level of Cadmium Exposure in Male Rats. Biol Trace Elem Res. 2016;170:146-51.
24. Zeneli L, Daci N, Paçarizi H, Daci L: The effects of taurine and melatonin on spermatogenesis in male rats exposed to cadmium. Int J Vitam Nutr Res, 2009;79: 328-31.