Turkish Journal of Medical Sciences

Volume 42 | Number 2 Article 9

1-1-2012

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Original Article

Turk J Med Sci
2012; 42 (2): 253-258
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E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-1002-9

The effect of soya bean meal on tibial articular cartilage growth in mice after suckling period: a histomorphometric and biochemical study

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Aim: To examine the effects of an oral supplement of soya bean meal on the histomorphometric alteration of tibia cartilage and the serum levels of calcium and alkaline phosphatase in 3-week-old female mice using a computer-assisted histomorphometric method.

Materials and methods: Forty immature BALB/c female mice were selected and divided into 4 groups. They were fed for 3 months with 4 different regimens: a low-protein regimen without soya bean meal, 23% protein without soya bean meal, 20% of the total protein being provided by soya bean meal, and 40% of the total protein being provided by soya bean meal. After 3 months, alkaline phosphatase and calcium determinations were performed. Using computer-assisted histomorphometric analysis, sections of the tibial plateaus were photographed. In order to measure the thickness and to count the number of chondrocytes in the middle part of the cartilage, haematoxylin and eosin stain was used. To measure the intensity of the articular cartilage, toluidine blue was also used.

Results: There were significant increases in the thickness of the cartilage, the number of chondrocytes in the serum calcium, and alkaline phosphatase activity in both soya bean-treated groups in comparison with the other groups. The concentration of the extracellular matrix in the groups with soya bean meal regimens was greater than that in the groups without soya bean meal regimens.

Conclusion: The present study suggests that a soya bean meal supplement can stimulate alkaline phosphatase production and increase the serum calcium, the number of chondrocytes, and the thickness of the cartilage in the middle part of the tibial plateau, in particular if started in childhood. Therefore, it is important to emphasise the effectiveness of a soya bean meal supplement to protect the joints.

Key words: Soya bean meal, articular cartilage, histomorphometric, biochemical, mice

Introduction

Articular cartilage consists of chondrocytes sparsely embedded within an abundant extracellular matrix, essentially composed of water, proteoglycans, collagens, and noncollagenous proteins. It is the swelling pressure of the partially hydrated proteoglycan gel, restrained by the inextensible collagen network, that gives cartilage its resilience and unique load-bearing properties (1). In the osteoarthritic joint, however, this equilibrium is disturbed in favour of proteoglycan catabolism, the loss of proteoglycans from the articular cartilage extracellular matrix leading to the deterioration of its biomechanical properties (2,3). Joint disease is a significant cause of lameness and disability

Received: 03.02.2010 – Accepted: 04.12.2010

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in humans (4), horses (5), and other domestic species (6). Exercise, nutrition, and joint loading can alter articular cartilage composition through alteration of chondrocyte metabolism (7,8).

The femorotibial joint is most commonly affected in osteoarthritis of the hamster, and involvement of one or more additional joints is also common (9).

A commercial avocado and soya bean mixture of unsaponifiables, and each component separately, could have a structure-modifying effect in osteoarthritis by inhibiting cartilage degradation and promoting cartilage repair (10). More recently, in studying chronic forms of arthritis, hamsters were maintained in the laboratory to an older age (11).

There is a great deal of interest in the use of plant material in osteoarthritic and rheumatoid arthritis disorders (12). For example, bromelain, an extract from the pineapple plant, demonstrated antiinflammatory and analgesic properties in clinical osteoarthritis trials (13). Avocado and soya bean oil contain a class of biologically active compounds classified as unsaponifiable lipids (avocado/soya unsaponifiables) (14). Avocado/soya unsaponifiables have recently been shown in various in vitro systems to partially reverse the effects of interleukin-13 on cultured chondrocytes by stimulating collagen synthesis and inhibiting the production of matrix metalloproteinases, interleukin-6, interleukin-8, and prostaglandin E_2 (1). The effect of avocado/soya bean extracts (piascledine) on the collagenolytic activity of cultured rabbit articular chondrocytes and human rheumatoid synovial cells has been studied and the results suggest a potential role for piascledine to limit the deleterious effects of interleukin-1 in osteoarticular diseases by reducing the capacity of this cytokine to stimulate collagenase production by synoviocytes and chondrocytes (15). Other studies showed that the alkaline phosphatase activity (ALP) was determined in the blood serum; there was a significant increase in serum ALP in all of the treated groups (16). Furthermore, soya's isoflavones increase the intestinal absorption of calcium (17). Studies on the effects of calcium on cartilage growth indicated that the activation of Ca receptors in the growth plate accelerates longitudinal bone growth by stimulating growth plate chondrogenesis (18).

The purpose of the present study was to examine the biochemical, histological, and histomorphometric changes of tibial cartilage in immature female mice following usage of soya bean meal.

Materials and methods

Animals and nutrition

A total of 40 female BALB/c mice, 21 days old and weighing 10-12 g, were provided by the Razi Institute (Karaj, Iran). They were maintained on 10/14-h light/dark cycles with 4 different diets and water provided ad libitum, and they were acclimatised in standard group housing (4 mice per cage) for a minimum of 1 week before use. This study was reviewed and approved by the Institutional Animal Care and Use Committee and was conducted in a facility that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animals were divided into 4 groups. The first group was fed a low-protein regimen containing 13% protein without soya bean meal for 3 months. In the second group, the regimen contained 23% protein without soya bean meal for 3 months. In the third and fourth groups, 20% and 40% of the daily total protein was provided by soya bean meal, respectively, for 3 months.

Biochemical analysis

After blood collection by cardiac puncture under general anaesthesia (intraperitoneal sodium pentobarbital at 50 mg kg^{-1}), the animals were killed. The blood was centrifuged and the serum was stored immediately at –2 °C for analysis. Sera were collected to evaluate in duplicate and stored at –70 °C. Serum levels of blood alkaline phosphatase were measured in advance using the photometric method, and the serum levels of blood calcium were measured using the cresolphthalein complexone method (19,20).

Handling of tissue

All of the animals were euthanatised with chloroform. Both hind limbs were removed and processed via histomorphometric evaluation of the tibial plateau.

In order to study the histological changes, the hind limbs were immersed overnight in 10% neutral buffered formalin to be fixed. The knees were decalcified for 72 h in a 1:1 mixture of 8 N formic acid and 1 N sodium formate (Kristiansen's solution) and then rinsed for 24 h with cold tap

water; then the protein contained in the tibial plateau was routinely processed and embedded. The knees were then mounted to allow 5-μm sagittal sections to be cut from the lateral to medial tibial plateaus. Approximately 6 serial sagittal sections were cut, beginning at the lateral region of the tibial plateau.

Histomorphometric image analysis

Computer-assisted histomorphometric analysis was conducted in a manner similar to that described by Shimizu et al. (21). Sections were photographed directly using a stereo microscope at 400× with the Microsoft system.

Cartilage thickness (5 μm) was determined from haematoxylin and eosin (H&E)-stained sections. The thickness was measured in the middle part of cartilage of the tibial plateau sections. Intensity of the toluidine blue staining and the total area (6.25×10^4 μm^2) of the articular cartilage were determined in each section. Furthermore, the intensity of toluidine blue staining in the articular cartilage was used as an index of the proteoglycan content (22). For determining the number of chondrocytes, the middle parts of the cartilage in the photographs were selected and the number of chondrocytes with a diameter of 6.25×10^4 μm^2 were counted (23).

Statistical comparisons were generated with analysis of variance (ANOVA) to analyse variance across different groups (for thickness and chondrocyte number) and with the Kruskal-Wallis test (for intensity of extracellular matrix), both at a significance level of $P < 0.05$.

Results

Effect of the different regimens on serum calcium

The final serum calcium level significantly increased in the soya bean meal regimens compared with the group with 23% protein without soya bean meal ($P < 0.05$) (Figure 1).

Effect of the different regimens on tibial cartilage

The histomorphometric study of the tibial cartilage via computerised image analysis showed a significant increase in the mean middle cartilage thickness of the proximal tibia in the soya bean meal regimen groups compared with the other groups ($P < 0.05$) (Table).

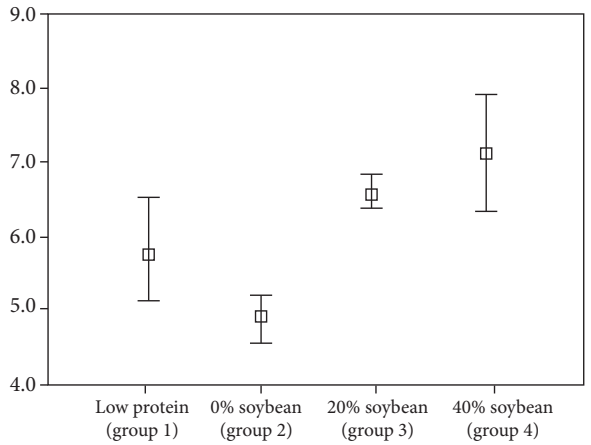

Figure 1. Relations between the mean level of serum calcium and different percentages of soya bean meal over the course of 3 months. Statistical analysis using 1-way ANOVA ($P < 0.05$) indicated that the mean level of serum calcium in group 4 (the group with the highest percentage of soya bean meal) was higher than in the group with 20% soya bean meal (group 3). In turn, the level of calcium in group 3 was higher than in the groups without soya bean meal (groups 1 and 2).

Effect of the different regimens on chondrocytes

The chondrocyte number within the cartilage regions of the proximal part of the tibia showed a significant increase in the soya bean meal regimen groups compared with the other groups ($P < 0.05$) (Table).

Effect of the different regimens on the intensity of extracellular matrix

The intensity of the toluidine blue staining showed a significant decrease in the low-protein, no-soya group compared with the other groups (Figure 2).

Effect of the different regimens on ALP

At the end of the experiment (after 3 months of using supplements), the serum levels of ALP were significantly higher in the soya bean meal regimen groups than in the other groups ($P < 0.05$) (Figure 3).

Discussion

Traditionally, histological analysis of cartilage was qualitative. Recent applications of image analysis systems to quantitative analysis of cartilage changes demonstrate the potential and acceptance of this technology (21,24,25), in which it was found that

Table. The relations between the mean thickness of the middle part of the cartilage of the tibial plateaus and the chondrocyte number within the cartilage regions of the proximal part of the tibia in groups with different percentages of soya bean meal for 3 months. Statistical analysis using 1-way ANOVA indicated that the mean thickness of the cartilage in group 4 (the group with 40% soya bean meal) was higher than in the group with 20% soya bean meal (group 3). Group 3, in turn, had a higher mean than the groups with 23% protein without soya bean meal (group 2) and with 13% protein without soya bean meal (group 1). Mean ± SE, N = 6, measurement range: μm, differing letters indicate significant differences at the level of $P < 0.05$.

Groups Parameter	1	2	3	4
Mean tibial cartilage (middle part)	18.33 ± 68.45 a	252.47 ± 18.74 a	405.74 ± 53.93 b	67.64 ± 53.21 b
Number of chondrocytes	6.86 ± 0.42 a	10.60 ± 0.75 b	11.44 ± 0.72 b	17.25 ± 5.30 c

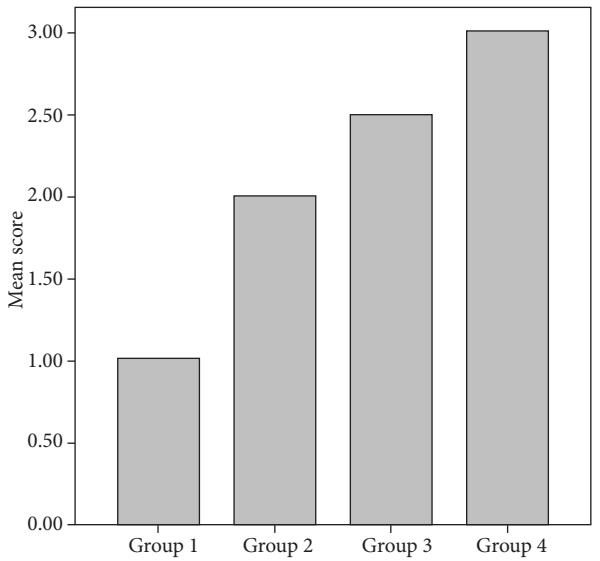

Figure 2. Relations between mean intensity of extracellular matrix and diet, showing a significant decrease in group 1 compared with the other groups in the middle part of the cartilage of tibial plateaus. Statistical analysis using Kruskal-Wallis test; $P < 0.05$.

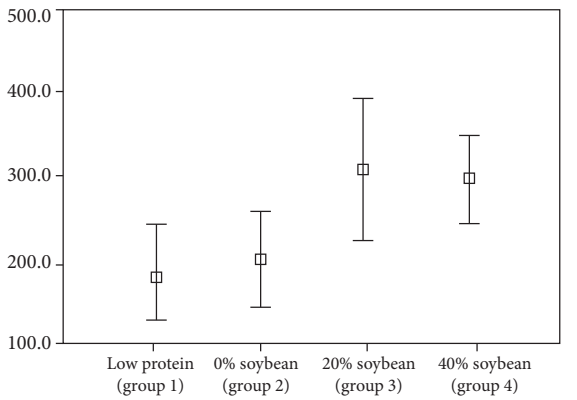

Figure 3. Relations between mean levels of serum alkaline phosphatase and diet in groups receiving different percentages of soya bean meal. Statistical analysis using 1-way ANOVA ($P < 0.05$) indicated that the mean serum levels of ALP were significantly higher in the soya bean meal regimen groups than in the groups without soya.

the determination of the cartilage staining intensity and area by computerised image analysis allowed a reliable and precise evaluation of the cartilage in a mouse model.

Results obtained in this study confirm the advantages of computerised, quantitative methodologies for the histomorphometric assessment of joint changes.

The role of soya protein and its isoflavones in the maintenance of health, such as the prevention of cardiovascular disease, certain types of cancer, and menopausal symptoms, is now widely recognised (26). In terms of bone, there are animal and human studies that have explored the role of soya in maintaining or increasing bone mass. In general, animal studies have shown that isoflavones in the context of soya protein have positive effects on bone marrow density (1,27). The findings of clinical trials have ranged from no significant changes to a slight increase in bone marrow density (28,29).

In the present study, the daily consumption of soya bean meal protein in immature female mice for 3 months led to a significant increase in the cartilage growth of the tibia, number of chondrocytes, and the presence of proteoglycan, especially in the high-dose regimen. The present study utilised an acceptable method that determines cartilage staining intensity and area by computerised image analysis to measure

the thickness in the medial part of the tibial plateau. Using toluidine blue staining provides further evidence of the partial preservation of articular cartilage integrity. Loss of toluidine blue staining was significantly observed in the low-protein regimen in both tibial plateaus after 3 months. Some investigators have reported that high-protein diets are associated with higher bone mineral density in the femoral neck. They have speculated that a high-protein diet may have a protective effect on hip bone mineral density over the long term. This notion, however, seems somewhat paradoxical, because high-protein diets, especially proteins rich in sulphur-containing amino acids, are known to increase urinary calcium, which may result in accelerated bone loss (30). Nonetheless, a counterargument has been made that protein-associated hypercalciuria is due to enhanced intestinal calcium absorption and not the breakdown of bone (17). Therefore, in this study, the total amount of protein was considered constant (except in the first group); however, the sources of provision in different diets were changed.

The tendency for increased (or reduced loss of) cartilage proteoglycan content in avocado/soya bean unsaponifiable (ASU)-treated animals may be the result of decreased catabolism and/or increased anabolism. It was recently suggested that many of the actions of ASUs may be mediated by an increase in expression of TGF-b, a potent stimulator of chondrocyte matrix production and an antagonist of the deleterious effects of interleukin-1. The results of this trial appear consistent with such a mechanism of action (31).

In this study, a significant increase in serum alkaline phosphatase was detected. Moreover, there was a correlation between cartilage thickness and ALP in the soya regimen groups after 3 months in immature female mice. Other studies showed that ALP was determined in the blood serum; there was a significant increase in serum ALP in all treated groups (16). Furthermore, one report showed that soya increases the intestinal absorption of calcium (17). Other studies showed that in the tibial growth plates, a progressive increase in ALP expression was seen in the chondrocytes and cartilage matrix, with the highest activity in the hypertrophic zone (32). Thus, the increase in serum alkaline phosphatase causes the increase in serum Ca, and in turn increases the thickness of the cartilage calcifying zone.

Studies have shown that calcium stimulates the uptake of SO_4 into the cartilage at physiological concentrations for ionised calcium. This effect can be blocked by puromycin, indicating that calcium stimulates the synthesis of proteoglycan (33). In this study, the concentration of serum calcium level increased following the usage of soya bean meal after 3 months in immature female mice. Therefore, the increase in serum calcium may have a positive effect on the synthesis of the extracellular matrix proteoglycan of the tibial cartilage.

In conclusion, the present experiment suggests that soya bean meal supplementation is capable of stimulating ALP production and reducing its loss in female mice after the suckling period. Furthermore, animals treated with higher rates of soya bean meal demonstrated greater thickness in the middle part of the tibial plateau, suggesting enhanced cartilage integrity, especially from an early age. Therefore, the effectiveness of soya bean meal supplementation during childhood to protect joints should be emphasised.

Acknowledgements

This study was supported by the Research Deputy of Islamic Azad University, Tehran Medical Branch; we thank him. We also gratefully acknowledge the authorities of the Faculty of Veterinary Medicine, University of Tehran, for allowing the use of their laboratory facilities.

References

1. Cake MA, Read RA, Guillou B, Ghosh P. Modification of articular cartilage and subchondral bone pathology in an ovine meniscectomy model of osteoarthritis by avocado and soya unsaponifiables (ASU). Osteoarthr Cart 2002; 8: 404-11.

2. Pelletier JP, Martel-Pelletier J, Howell DS. Etiopathogenesis of osteoarthritis. In: Koopman WJ, editor. Arthritis and allied conditions: A textbook of rheumatology. 13th ed. Baltimore (MD): Williams and Wilkins; 1997. p.1969-84.

3. Smith MM, Ghosh P. Osteoarthritis: Current status and future directions. APLAR J Rheumatol 1998; 2: 27-53.
4. Lohmander LS. What can we do about osteoarthritis? Arthritis Res 2000; 2: 95-100.
5. McIlwraith CW, Vachon A. Review of pathogenesis and treatment of degenerative joint disease. Equine Vet J Suppl 1988; 6: 3-11.
6. Vaughan S, Taylor TH. The pathophysiology and medical management of canine osteoarthritis. J S Afr Vet Assoc 1997; 68: 21-5.
7. Lane Smith R, Trindade MCD, Ikenoue T, Mohtai M, Das P, Carter DR et al. Effects of shear stress on articular chondrocyte metabolism. Biorheology 2000; 37: 95-107.
8. Lippiello L, Nardo JV, Harlan R, Chiou T. Metabolic effects of avocado/soy unsaponifiables on articular chondrocytes. eCAM 2007; 132: 1-13.
9. Otterness IG, Chang M, Burkhardt JE, Sweeney FJ, Milici AJ. Histology and tissue chemistry of tidemark separation in hamsters. Vet Pathol 1999; 36: 138-45.
10. Henrotin YE, Labasse AH, Jaspar JM, De Groote DD, Zheng SX, Guillou GB et al. Effects of three avocado/soybean unsaponifiable mixtures on metalloproteinases, cytokines and prostaglandin E2 production by human articular chondrocytes. Clin Rheumatol 1998; 17: 31-9.
11. Otterness IG, Eskra JD, Bliven ML, Shay AK, Pelletier JP, Milici AJ. Exercise protects against articular cartilage degeneration in the hamster. Arth & Rheum 1998; 41: 2068-76.
12. Ahmed S, Anuntiyo J, Malemud CJ, Haqqi TM. Biological basis for the use of botanicals in osteoarthritis and rheumatoid arthritis: a review. eCAM 2005; 2: 301-8.
13. Brien S, Lewith G, Walker A, Hicks SM, Middleton D. Bromelain as a treatment for osteoarthritis: a review of clinical studies. eCAM 2004; 1: 251-7.
14. Zorn J. New aspects in rheumatism therapy: experiences with a sitosterin preparation in chronic polyarthritis. Med Welt 1981; 32: 135-8.
15. Mauviel A, Loyau G, Pujol JP. Effect of unsaponifiable extracts of avocado and soybean (Piascledine) on the collagenolytic action of cultures of human rheumatoid synoviocytes and rabbit articular chondrocytes treated with interleukin-1. Rev Rhum Mal Osteoartic 1991; 58: 241-5.
16. Shiguemoto GE, Rossi EA, Baldissera V, Gouveia CH, de Valdez Vargas GM, de Andrade Perez SE. Isoflavone-supplemented soy yoghurt associated with resistive physical exercise increase bone mineral density of ovariectomized rats. Maturitas 2007; 57: 261-70.
17. Arjmandi BH, Smith BJ. Soy isoflavones' osteoprotective role in postmenopausal women: mechanism of action. J Nutr Biochem 2002; 13: 130-7.
18. Wu S, Palese T, Mishra OP, Delivoria-Papadopoulos M, De Luca F. Effects of Ca^{2+} sensing receptor activation in the growth plate. J FASEB 2004; 18: 143-5.
19. Banauch D, Brümmer W, Ebeling W, Metz H, Rindfrey H, Lang H et al. A glucose dehydrogenase for the determination of glucose concentrations in body fluids. Z Klin Chem Klin Biochem 1975; 13: 101-7.
20. Baginski ES, Marie SS, Clark WL, Zak B. Direct microdetermination of serum calcium. Clin Chim Acta 1973; 46: 46-54.
21. Shimizu C, Coutts RD, Healey RM, Kubo T, Hirasawa Y, Amiel D. Method of histomorphometric assessment of glycosaminoglycans in articular cartilage. *J Orthop Res* 2008; 15: 670-4.
22. Burdi AR. Toluidine blue-Alizarin red S staining of cartilage and bone in whole-mount skeletons in vitro. J Biotech & Histochjavascript:popRef('end-a1') 965; 40: 45-8.
23. Bobacz K, Erlacher L, Smolen J, Soleiman A, Graninger WB. Chondrocyte number and proteoglycan synthesis in the aging and osteoarthritic human articular cartilage. Ann Rheum Dis 2004; 63: 1618-22
24. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. J Bone Joint Surg 1971; 53: 523-37.
25. Milz S, Eckstein F, Putz R. The thickness of the subchondral plate and its correlation with the thickness of the uncalcified articular cartilage in the human patella. Anat Embryol (Berl) 1995; 192: 437-44.
26. Setchell KD. Soy isoflavones – benefits and risks from nature's selective estrogen receptor modulators (SERMs). J Am Coll Nutr 2001; 20: 354S-362S.
27. Verbruggen G. Chondroprotective drugs in degenerative joint diseases. J Rheum 2006; 45: 129-38.
28. Setchell KD, Lydeking-Olsen E. Dietary phytoestrogens and their effect on bone: evidence from in vitro and in vivo, human observational, and dietary intervention studies. Am J Clin Nutr 2003; 78: 593S-609S.
29. Cassidy A. Dietary phytoestrogens and bone health. J Br Menopause Soc 2003; 9: 17-21.
30. Kerstetter JE, O'Brien K, Insogna K. Dietary protein and intestinal calcium absorption. Am J Clin Nutr 2001; 73: 990-2.
31. Khalil DA, Lucas EA, Juma S, Smith BJ, Payton ME, Arjmandi BH. Soy protein supplementation increases serum insulin-like growth factor-I in young and old men but does not affect markers of bone metabolism. J Nutr 2002; 132: 2605-8.
32. Miao D, Scutt A. Histochemical localization of alkaline phosphatase activity in decalcified bone and cartilage. J Histochem Cytochem 2002; 50: 333-40.
33. Shulmana HJ, Opler A. The stimulatory effect of calcium on the synthesis of cartilage proteoglycan. Biochem Biophys Res Commun 1974; 59: 914-9.