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Cordycepin (3′-deoxyadenosine) Down-Regulates the Proinflammatory Cytokines in Inflammation-Induced Osteoporosis Model

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Abstract—The effect of cordycepin (3′-deoxyadenosine) on inflammation-induced osteoporosis (IMO) was studied in this paper. After the rats were treated orally with cordycepin (20 mg/kg), serum osteocalcin (OC), homocysteine (HCY), C-terminal cross-linked telopeptides of collagen type I (CTX), maleic dialdehyde (MDA), polymorphonuclear cells (PMN), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α), they were examined by ELISA or immunohistochemistry. The specimens from the liver were also processed for light microscopic examination. The IMO rats showed a significant increase in plasma CTX, MDA, PMN, IL-1β, TNF-α, and nitrate levels as well as a significant decrease in plasma OC. These changes were attenuated by cordycepin (20 mg/kg) supplementation in the IMO rats. Examination of the liver specimens revealed mononuclear cell infiltration in the portal areas in the IMO rats which was not detected in the cordycepin (20 mg/kg) rats. These results suggest that cordycepin may act as an anti-inflammatory agent in magnesium silicate-induced inflammation in osteoporosis.

KEY WORDS: cordycepin; IMO; proinflammatory cytokines; bone marrow cell.

INTRODUCTION

There is growing evidence that inflammation may be one of the causal factors of osteoporosis [1, 2]. This association was also observed clinically whereby the degree of osteoporosis was equivalent to the extent of inflammation.

Biochemical studies have demonstrated an elevation of proinflammatory cytokine tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in arthritic disease such as gouty arthritis, rheumatoid arthritis, and psoriatic arthritis [3, 4]. Inflammation may contribute to bone loss by affecting the bone remodeling process, favoring bone resorption activity by osteoclasts rather than bone formation activity by osteoblasts [5, 6]. Therefore, it is necessary to search for treatment modalities that not only mitigate osteoporosis but also having anti-inflammatory activities.

Cordycepin (3′-deoxyadenosine), a major bioactive component isolated from *Cordyceps militaris*, has multiple pharmacological activities, such as anti-inflammatory and immunomodulatory effects. Cordycepin prevents lipopolysaccharide (LPS)-induced airway neutrophilia in mice and effectively blocks LPS-induced expression of vascular adhesion molecule-1 (VCAM-1) in the human epithelial cell line A549 [7, 8]. Cordycepin inhibits interleukin-1β (IL-1β)-induced matrix metalloproteinase-1 (MMP-1) and MMP-3 expressions in rheumatoid arthritis synovial fibroblasts (RASFs) and significantly inhibits AP-1 activation [9].

Although several recent studies have suggested that cordycepin can exhibit anti-inflammation effect, to our knowledge, the effect of cordycepin on inflammation-induced osteoporosis was not described in the literature until now. It is worthwhile to investigate the cordycepin treatment toward both osteoporosis and inflammation. This study was performed to evaluate the possible protective effect of cordycepin on bone metabolism in an experimental inflammation-induced osteoporosis (IMO) model in

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which inflammation was induced by subcutaneous magnesium silicate to mimic the inflammatory and oxidative status that occurs with aging.

MATERIAL AND METHODS

Drugs

Cordycepin with 98 % purification was obtained following the extraction and separation using a column chromatographic method [10].

Animals

Healthy female adult Wistar rats (2 months old and weighing 225 ± 25 g) were used in the study. All animal experiments followed the guidelines published by the Ministry of Science and Technology of the People's Republic of China. Care was taken to minimize discomfort, distress, and pain to the animals.

Experimental Design

Inflammation-induced osteoporosis was induced by eight separate subcutaneous injections of magnesium silicate (3.2 g total per animal) in sterile saline on day 0 as previously described [11], and controls received an equal volume of saline. At ~6 weeks of age, the rats were randomized into four groups: controls ($n = 8$), IMO ($n = 8$), IMO + cordycepin (5 mg/kg) ($n = 8$), IMO + cordycepin (10 mg/kg) ($n = 8$), and IMO + cordycepin (20 mg/kg) ($n = 8$). Cordycepin was dissolved in distilled water and administrated orally two times daily using a feeding needle for 21 days, and the control group received double distilled water instead of cordycepin.

At the end of the experimental period, the animals were fasted overnight (18 h) and then sacrificed by decapitation; the blood was collected to be centrifuged at 3,000 rpm for 20 min, and the clear serum was separated for the measurement of serum osteocalcin (OC), homocysteine (HCY), and C-terminal cross-linked telopeptides of collagen type I (CTX) concentrations to evaluate osteoblastic activity. To determine inflammation and oxidative stress, levels of maleic dialdehyde (MDA), myeloperoxidase (MPO), IL-1β, and TNF-α were measured in the collected plasma. The liver was dissected out for histological examination.

Plasma Protein Measurements

The OC content was determined using an Osteocalcin EIA kit (Xinqidi Biological Technology, Inc., China) as described in the manufacturer's directions. Two OC antibodies were employed, each directed toward the N- or C-terminal OC molecule. HCY was measured by the use of an enzymatic fluorescence polarization immunoassay on an AxSYM analyzer (Abbott, Wiesbaden, Germany). CTX were quantified by enzyme-linked immunosorbent assay (ELISA) (Sunbio, Inc., China).

Measurement of Maleic Dialdehyde (MDA)

MDA was determined with thiobarbituric acid (TBA) using the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). The total protein content of the samples was analyzed using Coomassie blue assay (Nanjing Jiancheng Bioengineering Institute).

Measurement of Infiltration of PMN

The MPO activity was measured to assess the extent of polymorphonuclear cell (PMN) infiltration. The method of assaying MPO activity was based according to the guide of the assay kit (Nanjing Jiancheng Bioengineering Co., Ltd., China).

Measurement of IL-1β and TNF-α Level

The concentration of IL-1β and TNF-α was determined using a commercial ELISA kit (Shanghai Jinma Biological Technology, Inc., China) following the manufacturer's instruction.

Assessment of NO Production

Nitric oxide (NO) production was assessed by the measurement of the stable metabolic breakdown product nitrate (NO^-_3) as previously described [12, 13] on 18-h urine samples collected with the animals at the end of the experimental period.

Histological Examination of Liver

Liver specimens were fixed in 10 % buffered neutral paraformaldehyde solution, processed and embedded in paraffin. Thin paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E).

Statistical Analysis

Data were expressed as mean ± SEM, and the results were analyzed by ANOVA followed by Dunnett's t test. A p value of <0.05 was considered significant.

Table 1. Effects of Cordycepin on Plasma Proteins in IMO Rats

Groups	Serum OC (ng/mL)	Serum HCY (μmol/L)	Serum CTX (ng/mL)
Control	83.0±3.2*	7.7±1.0	75.6±4.2**
IMO	68.4±5.0	8.8±2.2	101.3±5.1
Cordycepin (20 mg/kg)	83.1±5.1*	9.3±1.1	79.0±10.2**
Cordycepin (10 mg/kg)	71.6±4.0	9.1±3.2	81.1±6.6*
Cordycepin (5 mg/kg)	69.0±4.8	8.9±2.1	86.3±3.0*

Values are mean±SEM. n=8. *p<0.05 versus IMO control; **p<0.01 versus IMO control.

RESULTS AND DISCUSSION

Several inflammatory diseases have been associated to bone resorption. Generalized osteoporosis and an increased risk of fracture are commonly observed in chronic inflammatory diseases [14, 15]. The first animal model of generalized osteoporosis resulting from inflammation that closely resembled the chronic inflammatory bone loss seen in human patients was the IMO model [16]. We found that animals with IMO that had been injected subcutaneously with magnesium silicate had increased circulating concentrations of inflammatory cytokines and TNF-α, when compared with controls. It is in agreement with the previous data [17, 18].

Effect of Cordycepin on Plasma Proteins

OC is known as serum markers reflecting osteoblast activities including bone formation and turnover [19]. The effects of treatment with cordycepin on OC level were shown in Table 1. The treatment of cordycepin (20 mg/kg) increased the OC level. However, the values of the groups treated with cordycepin (5 and 10 mg/kg) were not significantly higher than those of the IMO treated group. The result suggests that oral administration of cordycepin induces secretion of OC in a dose-dependent manner.

Some studies indicate that HCY stimulates osteoclasts and induces a dysbalance between osteoclasts and osteoblasts in favor of the osteoclasts [20, 21]. Compared with the IMO group, there were no significant differences in the increase of HCY content in cordycepin groups (Table 1).

Bone consists of a calcified organic matrix, which is composed of 90 % type I collagen [22]. During bone resorption, the molecule of type I collagen is degraded, and small fragments are liberated into the bloodstream. Higher CTX levels are associated with lower bone mineral density values in osteoporosis [23]. The serum levels of CTX were significantly higher in the IMO group than those in the other groups. All of the treatments significantly decreased the CTX level. However, the values of the group treated with cordycepin (20 mg/kg) were significantly lower than those of the other groups (Table 1). This finding suggests that cordycepin could increase the CTX level following magnesium-silicate-induced inflammation in an osteoporosis (IMO) model in a dose-dependent manner.

Effect of Cordycepin on Maleic Dialdehyde (MDA) Level

Peroxidation damage plays an important role in the progression of inflammation-mediated osteoporosis. It has

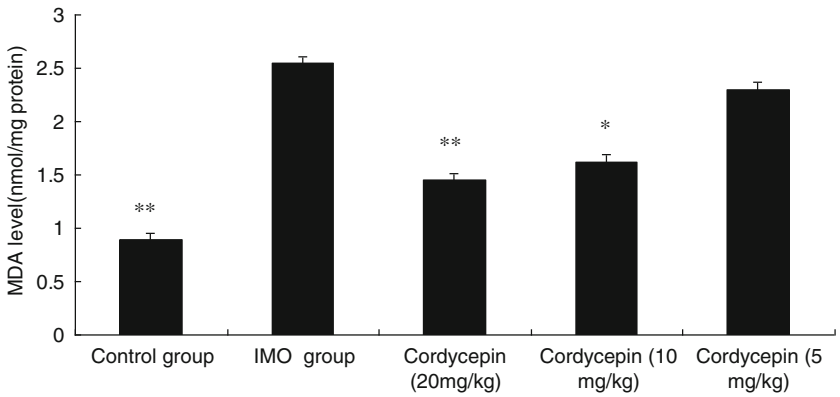

Fig. 1. Effect of cordycepin on MDA level. Values represent the mean±SEM. *p<0.05 versus IMO group. **p<0.01 versus IMO group.

Table 2. Effects of Cordycepin on MPO Activities

Different groups	(μmol g^{-1})
Control	0.99±0.21*
IMO	2.08±0.22
Cordycepin (20 mg/kg)	1.11±0.15*
Cordycepin (10 mg/kg)	1.66±0.21
Cordycepin (5 mg/kg)	1.87±0.20

Values are shown as means±SEM. *p<0.05 versus IMO group

been demonstrated that free radicals intervene in bone resorption promoting osteoclastic differentiation in such a manner that bone resorption is increased with oxidative stress [24]. Therefore, the antioxidant effects of cordycepin were investigated by measuring the MDA levels. The control animals showed low MDA levels; however, the MDA levels in the IMO group were significantly higher (p < 0.05). As shown in Fig. 1, the MDA level in the cordycepin (20 mg/kg) and cordycepin groups (10 mg/kg) were significantly lower than those in the IMO group (p < 0.01 and p < 0.05, respectively).

Effect of Cordycepin on Meloperoxidase (MPO) Activity

PMN infiltration is initiated after generation of inflammatory mediators. PMNs may contribute to secondary injury by causing microvessel occlusion and releasing oxygen radicals, cytolytic proteases, and proinflammatory cytokines, which may induce the neuronal damage

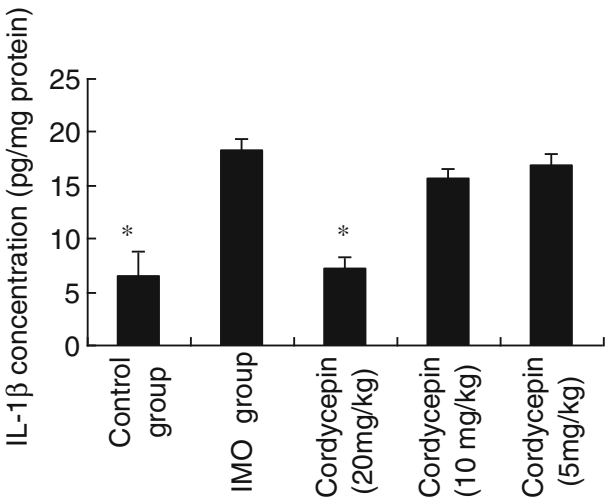

Fig. 2. Effect of cordycepin on IL-1β concentration. Values represent the mean±SEM. *p<0.05 versus IMO group.

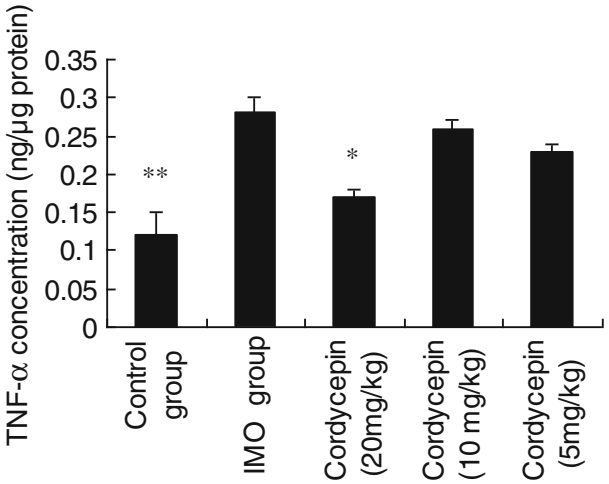

Fig. 3. Effect of cordycepin on TNF-α concentration. Values represent the mean±SEM. *p<0.05 versus IMO group.

[25, 26]. Hartl et al. reported that the reduction in the number of PMNs diminished postischemic tissue damage in the heart, intestine, lung, and liver [27]. The present study was undertaken to determine whether cordycepin reduces the number of PMNs in the serum. The activity of MPO was determined as an indicator of PMN migration. In this study, the MPO activity was relatively low in the control group and significantly increased in the IMO group. The treatment with cordycepin (20 mg/kg) significantly reduced the MPO activity (Table 2). The treatment with cordycepin (10 mg/kg) also reduced the MPO activity. However, it is not significant.

Effect of Cordycepin on IL-1β and TNF-α Level

The pathogenesis of osteoporosis is multifactorial, with many proinflammatory cytokines such as IL-1 and TNF released under osteoporotic conditions where they are involved in stimulating osteoclastic activity and regulation of bone resorption. These cytokines are thought to make an

Table 3. Effect of Cordycepin on NO Production

Different groups	(U g^{-1})
Control	4.77±1.20**
IMO	10.48±2.20
Cordycepin (20 mg/kg)	5.11±1.16**
Cordycepin (10 mg/kg)	7.66±1.20
Cordycepin (5 mg/kg)	8.88±1.20

Values are shown as means±SEM. **p<0.01 versus IMO group

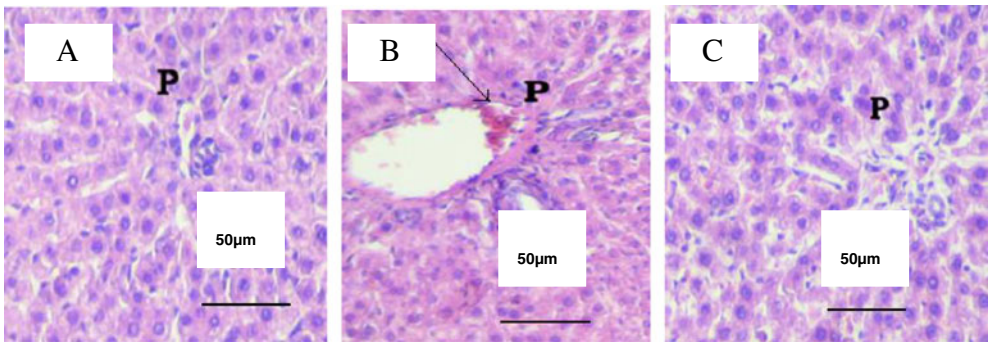

Fig. 4. Histological examination of liver, (H&E X400). **a** Section in the liver of the control rat showing branching and anastomosing cords of hepatocytes radiating from the central vein toward the portal area (*P*). **b** Section in the liver of the IMO rat showing mononuclear cellular infiltration (the *arrow*) in the portal area (*P*). **c** Section in the liver of the cordycepin (20 mg/kg) rat showing a picture similar to the control. Mononuclear cellular infiltration and congestion are not detected in the portal area (*P*).

important contribution in stimulating bone resorption and suppressing bone formation [28, 29]. We investigated the role of cordycepin in the pathogenesis of bone loss in an animal model of inflammation-induced osteoporosis. Figure 2 shows that IL-1β levels were significantly increased in the IMO rats. Treatment with both 20 and 10 mg/kg cordycepin resulted in a marked decrease in IL-1β levels compared with those in the IMO group ($p < 0.01$ and $p < 0.05$, respectively). In addition, the levels of TNF-α were significantly increased after magnesium silicate injection (Fig. 3). Cordycepin (20 mg/kg) suppressed magnesium silicate-induced TNF-α production ($p < 0.05$); however, this was not the case in the cordycepin-treated group (10 mg/kg). We demonstrated in this study that the administration of cordycepin decreased serum levels of IL-1β and TNF-α that are known to be produced by osteoblasts and induce bone resorption. Results from studies on cytokines have given us some insight on the mechanisms involved in the protection of cordycepin against osteoporosis.

Effect of Cordycepin on NO Production

Previous experiments have suggested that cytokine-induced NO production potentiates the effects of IL-1 and TNF on osteoclast and osteoblast activity *in vitro* [30]. In agreement with these data, NO^{-}_{3} levels had increased significantly in the IMO group when compared with controls ($p < 0.01$) (Table 3). This elevation was significantly inhibited by cordycepin (20 mg/kg). Treatment with both 10 and 5 mg/kg cordycepin caused a small decrease in NO production when compared with IMO, but this difference was not statistically significant ($p > 0.05$).

Histological Examination of Liver

Osteoporosis is a common complication of many types of liver disease. In the current study, histological examination of the liver of the IMO rats showed mononuclear cellular infiltration. Examination of sections of the livers of the control group revealed that the parenchyma was formed of classic hepatic lobules having the central veins in the middle and the portal tracts at the periphery. From the central vein, branching and anastomosing cords of hepatocytes radiate. The hepatocytes appeared polyhyderal in shape, with mildly vacuolated cytoplasm and rounded vesicular nuclei (Fig. 4a). In the IMO group, the most notable finding was the mononuclear cellular infiltration in the portal areas. Congested, dilated, and thickened blood vessels were also detected in the portal areas (Fig. 4b). In the cordycepin (20 mg/kg) group, the structure was similar to the control group. Mononuclear cellular infiltration and congestion were not detected (Fig. 4c).

CONCLUSION

In conclusion, our study showed that inflammation via magnesium silicate aggravated osteoporosis in the rat model. Cordycepin protected this rat model against bone loss. It is associated with the decreasing levels of HCY, CTX, MDA, MPO, IL-1β, and TNF-α in the serum as well as increased OC level. These results suggest that cordycepin may act as an anti-inflammatory agent in magnesium silicate-induced inflammation in osteoporosis.

REFERENCES

1. Yun, A.J., and P.Y. Lee. 2004. Maldaptation of the link between inflammation and bone turnover may be a key determinant of osteoporosis. *Medical Hypotheses* 63: 532–537.
2. Mitra, D., D.M. Elvins, D.J. Speden, and A.J. Collins. 2000. The prevalence of vertebral fractures in mild ankylosing spondylitis and their relationship to bone mineral density. *Rheumatology (Oxford)* 39(1): 85–89.
3. Moschen, A.R., A. Kaser, B. Enrich, et al. 2005. The RANKL/OPG system is activated in inflammatory bowel diseases and relates to the state or bone loss. *Gut* 54: 479–487.
4. Saidenberg-Kermanach, N., M. Cohen-Solal, N. Bessis, M.C. De Vernejoul, and M.C. Boissier. 2004. Role for osteoprotegerin in rheumatoid inflammation. *Joint Bone Spine* 71: 9–13.
5. Lorenzo, J. 2000. Interactions between immune and bone cells: new insights with many remaining questions. *Journal of Clinical Investigation* 106: 749–752.
6. Teitelbaum, S.L. 2000. Bone resorption by osteoclasts. *Science* 289: 1504–1508.
7. Kim, H., A.S. Naura, Y. Errami, J. Ju, and A.H. Boulares. 2011. Cordycepin blocks lung injury-associated inflammation and promotes BRCA1-deficient breast cancer cell killing by effectively inhibiting PARP. *Molecular Medicine* 17: 893–900.
8. Kim, H.G., B. Shrestha, S.Y. Lim, D.H. Yoon, W.C. Chang, D.J. Shin, et al. 2006. Cordycepin inhibits lipopolysaccharide-induced inflammation by the suppression of NFkappaB through Akt and p38 inhibition in RAW 264.7 macrophage cells. *European Journal of Pharmacology* 545: 192–199.
9. Noh, E.M., J.S. Kim, H. Hur, B.H. Park, E.K. Song, M.K. Han, et al. 2009. Cordycepin inhibits IL-1beta-induced MMP-1 and MMP-3 expression in rheumatoid arthritis synovial fibroblasts. *Rheumatology (Oxford)* 48: 45–48.
10. Ni, H., X.H. Zhou, H.H. Li, and W.F. Huang. 2009. Column chromatographic extraction and preparation of cordycepin from cordyceps militaris waster medium. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences* 877: 2135–2141.
11. Minne, H.W., J. Pfeilschifter, S. Scharla, S. Mutschelknauss, A. Schwarz, B. Krempien, and R. Ziegler. 1984. Inflammation-mediated osteopenia in the rat: a new animal model for pathological loss of bone mass. *Endocrinology* 115: 50–54.
12. Grabowski, P.S., A.J. England, R. Dykhuizen, M. Copland, N. Benjamin, D.M. Reid, and S.H. Ralston. 1996. Elevated nitric oxide production in rheumatoid arthritis: detection using the fasting urinary nitrate:creatinine ratio. *Arthritis and Rheumatism* 39: 643–647.
13. Ohshima, H., I.M. Brouet, T. Bandaletova, H. Adachi, S. Oguchi, S. Iida, Y. Kurashima, Y. Morishita, T. Sugimura, and H. Esumi. 1992. Polyclonal antibody against an inducible form of nitric oxide synthase purified from the liver of rats treated with propionibacterium acnes and lipopolysaccharide. *Biochemical and Biophysical Research Communications* 187: 1291–1297.
14. Deodhar, A.A., and A.D. Woolf. 1996. Bone mass measurement and bone metabolism in rheumatoid arthritis: a review. *British Journal of Rheumatology* 35: 309–322.
15. Andreassen, H., J. Rungby, J.F. Dahlerup, and L. Mosekilde. 1997. Inflammatory bowel disease and osteoporosis. *Scandinavian Journal of Gastroenterology* 32: 1247–1255.
16. Minne, H.W., J. Pfeilschifter, S. Scharla, et al. 1984. Inflammation-mediated osteopenia in the rat: a new animal model for pathological loss of bone mass. *Endocrinology* 115: 50–54.
17. Minne, H.W., J. Pfeilschifter, S. Scharla, S. Mutschelknauss, A. Schwarz, B. Krempien, and R. Ziegler. 1984. Inflammation-mediated osteopenia in the rat: a new animal model for pathological loss of bone mass. *Endocrinology* 115: 50–54.
18. Pfeilschifter, J., C. Wuster, M. Vogel, B. Enderes, R. Ziegler, and H.W. Minne. 1987. Inflammation-mediated osteopenia (IMO) during acute inflammation in rats is due to a transient inhibition of bone formation. *Calcified Tissue International* 41: 321–325.
19. Polak-Jonkisz, D., and D. Zwolinska. 1998. Osteocalcin as a biochemical marker of bone turnover. *Nephrology* 4: 339–346.
20. Koh, J.M., Y.S. Lee, Y.S. Kim, et al. 2006. Homocysteine enhances bone resorption by stimulation of osteoclast formation and activity through increased intracellular ROS generation. *Journal of Bone and Mineral Research* 21: 1003–1011.
21. Herrmann, M., N. Umanskaya, B. Wildemann, et al. 2008. Stimulation of osteoblast activity by homocysteine. *Journal of Cellular and Molecular Medicine* 12: 1205–1210.
22. Burgeson, R.E. 1988. New collagens, new concepts. *Annual Review of Cell Biology* 4: 552–577.
23. Guerrero, R., M.A. Diaz Martin, E.M. Diaz Diego, et al. 1996. New biochemical markers of bone resorption derived from collagen breakdown in the study of postmenopausal osteoporosis. *Osteoporosis International* 6: 297–302.
24. Sánchez-Rodríguez, M.A., M. Ruiz-Ramos, E. Correa-Muñoz, and V.M. Mendoza-Núñez. 2007. Oxidative stress as a risk factor for osteoporosis in elderly Mexicans as characterized by antioxidant enzymes. *BMC Musculoskeletal Disorders* 8: 124.
25. Widomski, D., D.J. Fretland, A.F. Gasiecki, and P.W. Collins. 1997. The prostaglandin analogs, misoprostol and SC-46275, potently inhibit cytokine release from activated human monocytes. *Immunopharmacology and Immunotoxicology* 19: 165–174.
26. Zoppo, G.J., G.W. Schmid-Schonbein, E. Mori, B.R. Copeland, and C.M. Chang. 1991. Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. *Stroke* 22: 1276–1283.
27. Hartl, R., L. Schurer, G.W. Schmid-Schonbein, and G.J. Zoppo. 1996. Experimental antileukocyte interventions in cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism* 16: 1108–1119.
28. Pacifici, R. 1996. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. *Journal of Bone and Mineral Research* 11: 1043–1051.
29. Poli, V., R. Balena, E. Fattori, A. Markatos, M. Yamamoto, H. Tanaka, et al. 1994. Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. *EMBO Journal* 13: 1189–1196.
30. Ralston, S.H., L.P. Ho, M.H. Helfrich, P.S. Grabowski, P.W. Johnston, and N. Benjamin. 1995. Nitric oxide: a cytokine-induced regulator of bone resorption. *Journal of Bone and Mineral Research* 10: 1040–1049.