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PHARMACOLOGY

ALENDRONATE PREVENTS DEVELOPMENT OF THE SKELETAL CHANGES INDUCED BY AZATHIOPRINE IN RATS

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Abstract: Immunosuppressive drugs are known to disturb bone remodeling. Azathioprine (AZA) is a potent immunosuppressive drug, but its effect on the skeletal system has not been reported so far. The aim of the present study was to investigate the effect of AZA on the rat bone remodeling, and the effect of alendronate on development of skeletal changes induced by AZA. The experiments were carried out on 3-month-old male Wistar rats, divided into the following groups: C – control rats (distilled water), AZA – azathioprine, ALN – alendronate, AZA + ALN – azathioprine and alendronate. Azathioprine (4 mg/kg *po*), alendronate (3 mg/kg *po*) and distilled water (2 ml/kg *po*) were administered once daily for 28 days. Bone remodeling was estimated based on quantitative and histomorphometric evaluation of the tibia and femur, and the mechanical properties of the femur and femoral neck. AZA at a dose of 4 mg/kg for 28 days induced bone remodeling disorders, inhibiting bone formation and mineralization. Alendronate prevented the development of skeletal changes caused by AZA administration, inhibiting bone resorption and increasing bone mineralization.

Keywords: azathioprine, alendronate, immunosuppression, bones, rats, osteoporosis

Azathioprine (AZA) is a potent immunosuppressive drug. Currently, it is used mainly in the treatment of autoimmune diseases, in particular non-specific inflammatory bowel diseases (1, 2). AZA is a pro-drug. In the body, it is subject of non-enzymatic transformation, occurring mainly in erythrocytes, to 6-mercaptopurine (6-MP) which is then metabolized intra-cellularly by hypoxanthine guanine phosphoribosyltransferase (HGPRT), thiopurine S-methyltransferase (TPMT), and xanthine oxidase (3, 4).

The immunosuppressive activity of AZA consists of competitive blocking of purine base synthesis by means of 6-thioguanine nucleotides (6-TGNs). TGNs are formed in the course of a multistage 6-mercaptopurine (6-MP) hepatic metabolism, with the participation of HGPRT inosine monophosphate dehydrogenase (IMPDH) and guanine monophosphate synthetase. Concurrently, inactivation of 6-MP by TPMT or xanthine oxidase takes place. TMPT transforms 6-MP into inactive 6-methyl-mercaptopurine (6-MMP), and xanthine oxidase into inactive 6-thiouric acid (3, 4).

6-TGNs have anti-proliferative activity. They inhibit the proliferation of cells and immune response by incorporation into DNA and inhibition of replication [3]. In T lymphocytes, the 6-TGNs reduce synthesis and content of purine nucleotides, mainly adenosine. They also induce the apoptosis of activated T lymphocytes, *via* blocking the activity of Rac-1 proteins and TRAIL expression, which leads to inhibited expression of the nuclear factor κB (NF-κB) and the STAT3 protein. 6-TGNs also block the expression of α4 integrin and inhibit the migration of lymphocytes to where the inflammatory process takes place (5, 6).

The mechanisms inhibiting immune response play a vital role in regulating functions of skeletal system cells, by influencing the expression of NF-κB and that of ligand of receptor activator of NF-κB (RANKL) as well as receptor activator of NF-κB (RANK). Apart from T lymphocytes, RANKL, like osteoprotegerin (OPG), are also produced by osteoblasts and bone marrow stromal cells, whereas RANK by osteoclasts. Binding RANKL to RANK activates NF-κB, which transmits to cell nucleus the

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signal responsible for differentiation of preosteoclasts into osteoclasts. OPG, binds RANKL and inhibits the activation of RANK (7-9). What is more, the activated T lymphocytes may inhibit osteoclastogenesis induced by RANKL (10, 11). Hence, AZA may disturb bone remodeling not only through inhibiting the activity of bone cells, but also through suppressive influence upon bone marrow stromal cells and immune system cells, leading to disturbances in the functioning of the RANKL/RANK/OPG system.

Disturbances in bone remodeling, leading to development of osteoporosis, have been observed after prolonged application of immunosupression with cyclosporin, tacrolimus, or glucocorticosteroids (12-14), whereas the influence of AZA on bone remodeling has not been studied so far. The aim of the study was to examine the influence of AZA on the skeletal system, as well as the influence of alendronate, a drug used in the treatment of osteoporosis, on the development of changes in the skeletal system, induced by AZA in rats.

EXPERIMENTAL

The study was performed with consent of the Local Ethics Committee in Katowice, on 32 3-month-old male Wistar rats of initial body mass

Table 1. Body mass gain and bone quantity parameters.

Parameters		Groups			
		C	AZA	ALN	AZA + ALN
Initial body mass [g]		267.3 ± 6.5	260.4 ± 4.2	260.2 ± 5.5	263.2 ± 5.4
Body mass after 28 days [g]		323.5 ± 6.0	312.0 ± 5.1	308.5 ± 6.5	310.2 ± 6.5
Body mass gain after 28 days [g]		56.3 ± 2.8	51.6 ± 2.2	48.2 ± 2.5	47.0 ± 2.5 *
Bone mass [mg]	Tibia	612.93 ± 11.29	575.59 ± 8.28 *	606.40 ± 8.74	622.11 ± 10.18 ^{a}
	Femur	868.50 ± 10.75	821.21 ± 15.88 *	855.46 ± 11.99	877.08 ± 14.44 ^{a}
	L-4 Vertebra	296.30 ± 7.29	274.24 ± 5.42 *	291.01 ± 5.87	287.10 ± 4.51
Bone mass/body mass ratio [mg/100 g of body mass]	Tibia	188.62 ± 1.33	184.59 ± 1.72	193.91 ± 1.94 *	196.60 ± 1.53 ** ^{aaa}
	Femur	267.41 ± 1.28	263.17 ± 1.74	273.55 ± 2.39	277.26 ± 3.34 * ^{aa}
	L-4 Vertebra	91.16 ± 1.37	87.93 ± 1.29	93.05 ± 1.50	90.80 ± 1.41
Bone mineral mass [mg]	Tibia	248.15 ± 5.21	234.91 ± 2.56 *	252.44 ± 2.97	262.50 ± 5.80 ^{aa}
	Femur	347.18 ± 7.26	332.90 ± 5.21	343.40 ± 4.01	365.54 ± 7.45 ^{aa}
	L-4 Vertebra	91.41 ± 2.15	85.41 ± 1.43 **	94.40 ± 0.83	96.53 ± 2.44 ^{aa}
Bone mineral mass/bone mass ratio [mg/100 mg of bone mass]	Tibia	40.48 ± 0.33	40.83 ± 0.28	41.66 ± 0.44	42.17 ± 0.34 ** ^{aa}
	Femur	39.96 ± 0.56	40.57 ± 0.33	40.16± 0.25	41.66 ± 0.19 * ^{a}
	L-4 Vertebra	30.86 ± 0.20	31.20 ± 0.64	32.52 ± 0.61 *	33.60 ± 0.45 ** ^{a}
Calcium content [mg/g of bone mineral]	Tibia	324.55 ± 3.92	309.42 ± 4.16 *	329.34 ± 6.70	327.95 ± 7.46 ^{a}
	Femur	327.66 ± 4.15	311.68 ± 3.79 *	328.54 ± 3.26	323.98 ± 3.29 ^{a}
	L-4 Vertebra	319.45 ± 4.77	306.83 ± 5.99	322.38 ± 5.48	323.98 ± 5.55 ^{a}

C – Control rats, AZA – rats receiving azathioprine (4 mg/kg), ALN – rats receiving alendronate (3 mg/kg), AZA + ALN – rats receiving azathioprine (4 mg/kg) and alendronate (3 mg/kg). Results are presented as the mean values ± SEM (n = 8). * – Significantly different from the control group (C): * – $p < 0.05$, ** – $p < 0.01$. ^{a} – Significantly different from the rats receiving AZA: ^{a} – $p < 0.05$, ^{aa} – $p < 0.01$, ^{aaa} – $p < 0.001$.

251–282 g. Rats were divided into four groups (n = 8): C – control rats receiving distilled water (2 mL/kg *po*), AZA – rats which were administered azathioprine (4 mg/kg *po*), ALN – rats which were administered alendronate (3 mg/kg *po*), AZA + ALN – rats which were administered azathioprine (4 mg/kg *po*) and alendronate (3 mg/kg *po*). Azathioprine – Azathioprine, tablets 50 mg (Zakłady Chemiczno-Farmaceutyczne VIS) and alendronate sodium, substance (Polpharma S.A.) were used in the study. Azathioprine, alendronate and distilled water were administered *via a* gastric tube once daily, for 28 days. The next day after the last drug administration the animals were killed and the tibia (left and right), femur (left and right), L-4 vertebra and liver have been isolated.

Bone remodeling was estimated based on quantitative and histomorphometric evaluation of the tibia and the femur and the mechanical properties of the whole femoral bone and femoral neck. The investigations of quantitative parameters included: length and diameter of the tibia and femur, bone mass directly after isolating and bone mass/body mass ratio, bone mineral mass (ash) assayed by mineralization method (15) and bone mineral mass/bone mass ratio, as well as calcium content in the bone mineral assayed by colorimetrical method with the use of chemical analyzer Pointe-180 Plus (by Pointe Scientific).

Investigations of histomorphometric parameters have been made in the tibial diaphysis (transverse growth and the osteoid width from the periosteum and endosteum side, transverse cross-section area of the cortical bone, entire diaphysis and the marrow cavity, marrow cavity area/entire diaphysis area ratio) and in the femur (width of trabeculae in the distal epiphysis and metaphysis). The estimation of histomorphometric parameters was carried out on histological preparations prepared from sections of non-decalcified bones, obtained as a result of cutting tibial diaphysis and distal femoral epiphysis. In order to determine transverse growth, the rats were given tetracycline hydrochloride twice. Histomorphometric estimation of the specimens was carried out using a set including Optiphot-2 Nikon microscope with visible and ultraviolet light range, an RGB video camera (Cohu), and computer with Lucia G 4.51 software for digital histological measurements (15, 16).

The studies of bone mechanical properties of the entire femur were performed using a three-point bending test. Extrinsic stiffness, ultimate load and breaking load, as well as deformation caused by the ultimate and breaking loads were assessed. Mechanical properties of the femoral neck were studied using a compression test. The load causing the fracture of the femoral neck was determined (17, 18).

The results are presented as the arithmetical mean ± SEM. Statistical estimation was carried out using Statistica 9 software. Non-parametric Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used. The results obtained in all groups of rats were compared with those of the control group. Moreover, the results of AZA + ALN group were compared to those of the AZA group. The differences were regarded as statistically significant at $p < 0.05$.

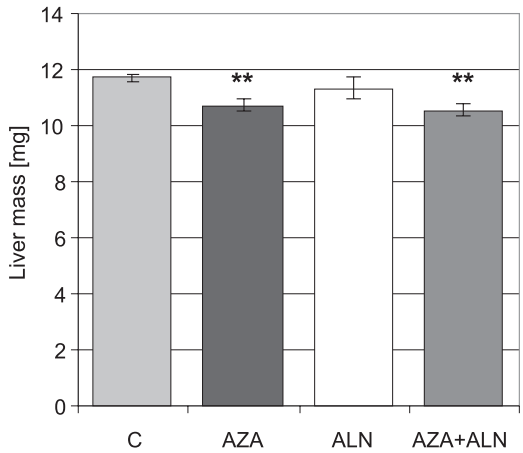

Figure 1. Liver mass. Abbreviations see Table 1. Results are presented as the means ± SEM (n = 8). * – Significantly different from the control group (C): ** – $p < 0.01$

RESULTS

Bone quantity parameters and body mass gain

AZA caused statistically significant, in reference to group C, reduction of femur mass (by 5.4%), tibia mass (by 6.1%), and vertebra mass (by 7.4%), as well as reduction of the mass of mineral substances in tibia (by 5.3%) and vertebra (by 6.6%). Also calcium content got reduced in femur (by 4.9%) and in tibia (by 4.7%). However, it did not cause significant changes in bone mass/100 g of body mass, nor the mass of mineral substances/100 mg of bone mass ratio (Tab. 1), as well as in the length and diameter of the femur (results non shown). AZA also caused statistically significant reduction of liver mass in comparison with group C, (Fig. 1).

Alendronate, administered to rats receiving azathioprine (AZA + ALN) caused statistically significant increase, in relation to rats administered only

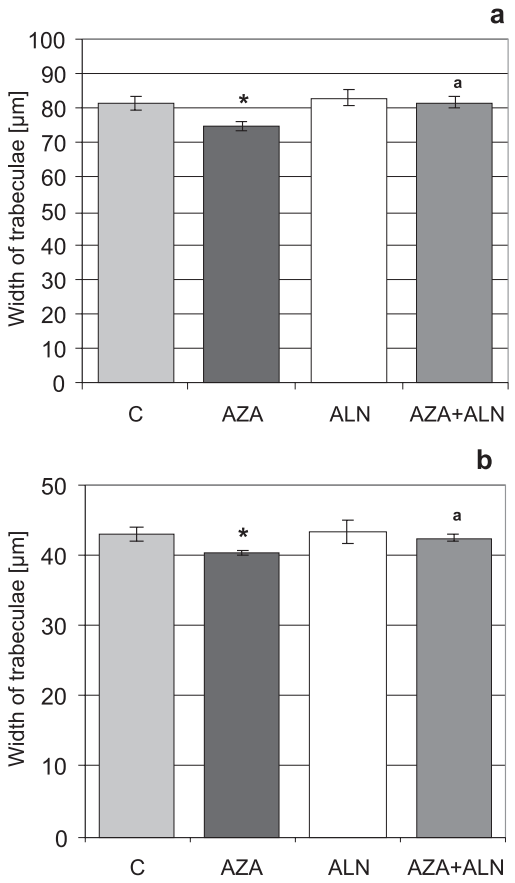

Figure 2. Width of trabeculae in the distal epiphysis (**a**) and methaphysis (**b**) of the femur. Abbreviations see Table 1. Results are presented as the means ± SEM (n = 8). * – Significantly different from the control group (C): * – $p < 0.05$. [a] – Significantly different from the rats receiving AZA: [a] – $p < 0.05$

AZA, of tibia mass (by 8.1%) and femur mass (by 6.8%), as well as mass of those bones/100 g of body mass, by 6.5% and 5.4%, respectively, the mass of mineral substances in tibia (by 11.7%), femur (by 9.8%) and vertebra (by 13.0%), and the mass of mineral substances/100 mg of mass of those bones, by 3.3%, 2.7%, and 7.7%, respectively. Calcium content was also increased in tibia (by 6.0%), femur (by 3.9%), and vertebra (by 5.6%). Alendronate, administered to rats together with AZA, caused statistically significant increase, in relation to rats from group C, of tibia mass (by 4.2%), femur mass (by 3.7%), mass of mineral substances/100 mg of tibia mass (by 4.2%), femur mass (by 4.2%) and vertebra mass (by 8.9%), and statistically significant reduction (by 16.5%) of rat body mass gain (Tab. 1).

Histomorphometric parameters of the tibia and of the femur

AZA caused statistically significant, in reference to group C, reduction of transverse growth and osteoid width from the periosteum side, by 12.1% and 13.5%, respectively, and from the endosteum side by 11.3% and 10.2%, respectively, with simultaneous, statistically significant, reduction of the transverse cross-section area of cortical bone (by 6.6%) and the entire diaphysis of tibia (by 4.7%, Tab. 2), as well as significant reduction of bone trabeculae width, in epiphysis (by 7.9%, Fig. 2a) and in distal metaphysis of femur (by 6.1%, Fig. 2b).

Alendronate, administered to rats receiving azathioprine (AZA + ALN) caused statistically significant increase, in relation to rats administered only AZA, of tibia diaphysis area (by 7.3%) and osteoid width from the periosteum side (by 12.7%) and from the endosteum side, by 10.1% and 9.1%, respectively, with simultaneous, statistically insignificant, increase of area of cortical bone (by 5.3%) and the entire diaphysis of tibia (by 3.5%, Tab. 2) as well as significant increase of bone trabeculae width, in epiphysis (by 9.0%, Fig. 2a) and in distal metaphysis of femur (by 5.0%, Fig. 2b).

Mechanical properties of the whole femur and the femoral neck

AZA caused statistically significant, in reference to group C, reduction of extrinsic stiffness (by 11.4%), ultimate load (by 11.8%) and fracture load of femur diaphysis (by 10.7%), as well as reduction of load causing fracture of femoral neck, by 11.0% (Tab. 3).

Alendronate, administered to rats receiving azathioprine (AZA + ALN) caused statistically significant, in reference to rats receiving AZA only, increase of the extrinsic stiffness (by 8.3%), ultimate load (by 12.0%), and fracture load of femur diaphysis (by 14.1%). It also caused a statistically insignificant increase (by 3.8%) of the load causing fracture of femoral neck (Tab. 3).

DISCUSSION

AZA ranks among the most effective immunosuppresive drugs, used in treatment of steroid-resistant, and steroid-dependent forms of non-specific inflammatory bowel diseases (IBD). The efficacy of AZA in IBD treatment depends upon the enzymatic activity of TPMP and concentration of the 6-TGNs. In case of low activity of TPMP, reduction of 6-MP methylation to inactive metabolite, and preferential metabolism of 6-MP to 6-TGNs occurs, which leads

to increase of suppressive effect on bone marrow cells. Among the most prominent unwanted effects, occurring in approximately 25% of patients treated with AZA, there are myelosuppression and hepatoxicity (1, 2). The results obtained in the study indicate that AZA, applied in doses that do not impair the healing of intestinal anastomosis in rats (19), also disturbs the bone remodeling and may, in case of prolonged therapy, induce the development of osteoporosis.

Correct bone remodeling depends upon maintaining a balance between bone resorption by osteoclasts, and bone formation by osteoblasts. Bone remodeling takes place both in bones with compact structure, and with trabecular structure, and is indispensable for maintaining proper mass and mechanical endurance of bones (20). AZA disturbed the remodeling of bones having compact and trabecular structure, by inhibiting bone formation. In bones with compact structure, it caused substantial reduction of osteoid width, and transverse growth from periosteum and endosteum side, with simultaneous reduction of cortical bone area and the entire diaphysis area of the tibia, while in trabecular bones, reduction of bone trabeculae width occured, in epiphysis and metaphysis of femur. Moreover, AZA caused reduc-

Table 2. Histomorphometric parameters of the tibia.

Parameters		Groups			
		C	AZA	ALN	AZA + ALN
Transverse cross-section area cortical [mm^2]	bone marrow	3.755 ± 0.048	3.509 ± 0.027 **	3.681 ± 0.092	3.690 ± 0.100
	cavity whole	0.940 ± 0.048	0.968 ± 0.037	0.911 ± 0.030	0.940 ± 0.037
	diaphysis	4.695 ± 0.047	4.477 ± 0.042 *	4.592 ± 0.088	4.630 ± 0.117
Transverse cross-section marrow cavity/diaphysis area ratio		0.200 ± 0.010	0.216 ± 0.007	0.199 ± 0.007	0.203 ± 0.006
Transverse growth [μm]	periosteal	56.67 ± 1.92	49.79 ± 1.10 *	56.16 ± 2.20	53.45 ± 0.88 ^{a}
	endosteal	38.08 ± 1.57	33.78 ± 0.52 *	34.63 ± 0.46	37.19 ± 0.65 ^{a}
Osteoid width [μm]	periosteal	14.81 ± 0.14	12.81 ± 0.52 **	14.91 ± 0.38	14.44 ± 0.35 ^{a}
	endosteal	9.68 ± 0.21	8.69 ± 0.26 *	9.55 ± 0.23	9.48 ± 0.26 ^{a}

Abbreviations see Table 1. Results are presented as the means ± SEM (n = 8). * – Significantly different from the control group (C): * – $p < 0.05$, ** – $p < 0.01$. ^{a} – Significantly different from the rats receiving AZA: ^{a} – $p < 0.05$, ^{aa} – $p < 0.01$.

Table 3. Mechanical properties of the whole femur and the femoral neck.

Parameters		Groups			
		C	AZA	ALN	AZA + ALN
Extrinsic stiffness [mm/N]		263.91 ± 6.76	233.89 ± 3.72 *	262.84 ± 5.29	253.37 ± 5.29 ^{a}
Load [N]	maximum	77.95 ± 1.77	68.78 ± 1.00 **	80.39 ± 4.61	77.05 ± 2.51 ^{a}
	fracture	72.84 ± 2.56	65.02± 1.12 *	75.83 ± 4.46	74.18± 1.61 ^{aa}
Displacement [mm]	at maximum load	0.222 ± 0.010	0.228 ± 0.032	0.214± 0.027	0.230 ± 0.016
	at fracture load	0.281± 0.011	0.297± 0.027	0.272 ± 0.029	0.294± 0.013
Load at fracture of the femoral neck [N]		108.87 ± 3.79	96.90 ± 1.97 *	111.67 ± 2.61	100.61 ± 1.40

Abbreviations see Table 1. Results are presented as the means ± SEM (n = 8). * – Significantly different from the control group (C): * – $p < 0.05$, ** – $p < 0.01$. ^{a} – Significantly different from the rats receiving azathioprine: ^{a} – $p < 0.05$, ^{aa} – $p < 0.01$.

tion of bone mass and the mass of mineral substances, as well as calcium content, which may indicate also intensified resorption. However, at the absence of significant changes of the marrow cavity area, and in the ratio of marrow cavity area to the entire bone diaphysis area, the reduction of bone mass and mineral substances mass indicates inhibition of bone formation. Disturbances in macroarchitecture (reduction of cortical bone and bone diaphysis area), and microarchitecture (thinning of bone trabeculae), caused by AZA, led to deterioration of mechanical endurance of bones. As regards elastic deformation region, reduction of extrinsic stiffness has been observed, reduction of ultimate load and breaking load in the plastic deformation region. AZA also reduced the mechanical endurance of femoral neck.

The importance of purine synthesis inhibition in cells of osteoblastic and osteoclastic lines has not been explained so far. However, on the basis of the recognized mechanisms that regulate bone remodeling, the disturbances found after the use of AZA may result from impairing the functions of RANKL/RANK/OPG system in osseous cells. Inhibition of the synthesis of purines in cells of the osteoblastic and osteoclastic lines may lead to disturbed regulation of osteoclastogenesis and bone resorption by osteoclasts. Inhibition of osteoblastic activity may lead to inhibition of bone formation, but also to disturbances of osteoclastogenesis regulation by affecting production of proresorptive cytokines (RANKL and M-CSF) (9, 21). The bone remodeling disturbances found after the administration of AZA may be due to impaired functions of osteoblastic cells. One cannot exclude also the direct inhibiting influence of AZA upon the synthesis of purines in cells of the osteoclastic cell line, leading (as is the case in T lymphocytes) to blocking the NF-κB activation and intensification of osteoclast apoptosis (5). The demonstrated disturbances in bone remodeling, induced by AZA require, nevertheless, further studies, which would enable explanation for their molecular mechanisms.

In order to study the possibility of counteracting the development of bone remodeling disorders induced by AZA, alendronate was administered at a dose of 3 mg/kg. The dose was chosen based on previous animal experimental studies, in which the antiresorptive activity of alendronate administered at doses of 1–5 mg/kg was demonstrated (22-24). Alendronate prevented the development of bone remodeling disturbances induced by AZA in the compact and cancellous bone. In compact bone, the alendronate decreased the area of marrow cavity and the proportion between marrow cavity area and tibia diaphysis area, whereas in trabecular bone, it increased width of bone trabeculae epiphysis and metaphysis of the femur. Moreover, the mass of mineral substances/100 mg of bone mass increased statistically significantly in all bones examined, reaching higher values than in control rats. Those changes were the consequence of antiresorptive activity of alendronate, and led to improved mechanical endurance of bones. In rats exposed to the immunosuppressive activity of AZA, alendronate caused significant increase of extrinsic stiffness, as well as ultimate load and breaking load. To a lesser extend, it reduced influence of AZA the mechanical properties of femoral neck.

Antiresorptive activity of alendronate is connected with inhibiting the synthesis of cholesterol and izoprenoid lipids in the mevalonate metabolism pathway. Izoprenoid lipids are indispensable for prenylation of cytoplasmic proteins having the properties of GTP-ases. Absence of prenylation of GTP-ases in osteoclasts results in inhibiting their function and bone resorption. Inhibition of bone resorption by alendronate causes a shift of balance in bone turnover, for the benefit of bone formation (25-27). Hence rats receiving alendronate together with AZA demonstrated significant increase of osteoid width and transverse growth from the periosteum and endosteum side, and increase of bone trabeculae in the femoral epiphysis and metaphysis. Moreover, in the ratio of bone mass to body mass increased significantly, reaching values much higher than those determined for control rats. Those results are indicative of intensified process of bone formation, and have been confirmed by *in vitro* studies. Im et al. have demonstrated that alendronate caused increased expression of BMP-2 genes, collagen type I, and osteocalcin in cultures of human osteoblastic cells (28). The stimulative influence of alendronate on the expression of genes, which are of key importance for differentiation of osteoblasts in human bone marrow cells has also been demonstrated by von Knoch et al. (29). The results obtained in the study for the first time provide data concerning the influence of AZA on bone remodeling *in vivo*. They indicate that bone remodeling disturbances may result from the suppressive influence of AZA on osseous cells of the osteoblastic line, and provide basis for the use of alendronate in prevention of bone remodeling disorders induced by AZA.

CONCLUSION

AZA, administered for 4 weeks in a dose of 4 mg/kg, disturbed bone remodeling in rats, by inhibiting bone formation.

Alendronate prevented the development of AZA-induced disorders in bone remodeling in rats inhibiting bone resorption and increasing bone mineralization.

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