Thalidomide affects the skeletal system of ovariectomized rats

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Abstract:

Apart from having written an inglorious chapter in the history of medicine, thalidomide is currently being intensely studied because of its multidimensional activity. The aim of this study was to examine the effects of thalidomide on the skeletal system in ovariectomized and non-ovariectomized rats.

The experiments were carried out with female Wistar rats, divided into eight groups: sham-operated control rats; sham-operated rats receiving thalidomide at doses of 15, 30 or 60 mg/kg, *po*; ovariectomized control rats; ovariectomized rats receiving thalidomide at doses of 15, 30 or 60 mg/kg, *po*. The drug was administered for 4 weeks.

Body mass gain and the mass of the uterus, liver, spleen and thymus were studied. Macrometric parameters and content of mineral substances, calcium and phosphorus in the femur, tibia and L-4 vertebra and histomorphometric parameters of the femur and tibia were examined. In the femur, the mechanical properties of the whole bone and of the femoral neck were examined.

Thalidomide did not affect the skeletal system of the non-ovariectomized rats.

Bilateral ovariectomy induced osteoporotic skeletal changes in mature female rats. The effects of thalidomide on the skeletal system of ovariectomized rats depended on the dose used. With a dose of 15 mg/kg, *po*, thalidomide counteracted some osteoporotic changes induced by estrogen deficiency. With a dose of 60 mg/kg, *po*, thalidomide intensified the destructive effects of estrogen deficiency on the rat skeletal system.

Key words:

thalidomide, bone, osteoporosis, ovariectomy, rats

Introduction

Apart from having written an inglorious chapter in the history of medicine, thalidomide is currently one of the most intensely studied drugs because of its multidimensional activity. Thalidomide has been approved for the treatment of multiple myeloma and erythema nodosum leprosum. Clinical trials are currently being conducted for its use in treating inflammatory diseases, various neoplastic diseases, complications of human immunodeficiency virus infection, chronic

graft-versus-host disease and numerous dermatological conditions, among others [1, 13, 19, 23, 25, 27, 29, 30, 37, 44].

Multiple myeloma is often accompanied by pathologic fractures, severe bone pain and hypercalcemia [38]. Thalidomide has been reported to improve the status of multiple myeloma patients, among others, by controlling lytic bone lesions [17, 39, 40, 41]. Several reports focusing on the mechanism by which thalidomide can suppress excessive bone resorption in patients with multiple myeloma have recently been published [2, 20, 39–41]. It is proposed that thalidomide's

ability to reduce osteolytic bone destruction in multiple myeloma is due to direct inhibition of osteoclastogenesis, and to antineoplastic effects on the myeloma cells themselves [39]. It was demonstrated that in some patients thalidomide may also inhibit bone formation [40, 41].

As far as we know, no detailed studies have yet been performed which focus on the effects of thalidomide on the skeletal system of humans or experimental animals. Almost all reports concerning the effects of thalidomide on bones refer to the teratogenic effects on the osseous tissue in infants born to women who used thalidomide during pregnancy, and in the fetuses of experimental animals [11, 24]. Sparse clinical and experimental data regarding the effects of thalidomide on skeletal tissue substantiate the necessity to examine the drug's effects on bone remodeling. Therefore, in the present study, we decided to investigate the effects of thalidomide on the skeletal system in non-ovariectomized and ovariectomized rats.

Materials and Methods

The experiments were carried out with three-month-old female Wistar rats, which were given tap water to drink and fed a standard laboratory diet *ad libitum*. Permission for the animal experiments was granted by the Local Ethics Commission, Katowice, Poland.

The animals were divided into eight groups ($n = 7$) according to the type of surgical operation they underwent and the dose of thalidomide they were given: Group I – control sham-operated (SHAM) rats; Group II – sham-operated rats + thalidomide 15 mg/kg, *po*; Group III – sham-operated rats + thalidomide 30 mg/kg, *po*; Group IV – sham-operated rats + thalidomide 60 mg/kg, *po*; Group V – control ovariectomized (OVX) rats; Group VI – ovariectomized rats + thalidomide 15 mg/kg, *po*; Group VII – ovariectomized rats + thalidomide 30 mg/kg, *po*; Group VIII – ovariectomized rats + thalidomide 60 mg/kg, *po*.

Thalidomide (purchased from Sigma) was administered to the rats in groups II–IV and VI–VIII once daily for 4 weeks as a suspension in distilled water, in a volume of 1 ml/kg. The control sham-operated rats (group I) and ovariectomized rats (group V) were given distilled water in the same volume of 1 ml/kg, *po*. Administration of thalidomide or distilled water

started 2 days after the surgery. Twenty-four hours prior to the first administration, and on the last day of administration of the drug or distilled water, the animals were given tetracycline hydrochloride (20 mg/kg, *ip*) to mark the calcification front. Tetracycline hydrochloride is a histomorphometric fluorescence marker [9].

Bilateral ovariectomy or sham-operation were performed under ether anesthesia. A longitudinal incision was made inferior to the rib cage on the dorsolateral body wall. The ovaries of rats in groups V–VIII were exteriorized, ligated and excised. The sham-operated rats (groups I–IV) had their dermal integuments, muscles and peritoneum sectioned, but underwent no excision of the ovaries.

After 4 weeks of drug administration, all of the animals were sacrificed. The right and left femoral and tibial bones, L-4 vertebra, and the uterus, liver, spleen and thymus were isolated and weighed. The macro-metric parameters (length, diameter of the diaphysis at the mid-length and diameter of the epiphysis) in the femur and tibia were assessed.

The femoral and tibial bones were used to prepare histological specimens. The histological specimens were prepared and measured as previously described [8]. From the tibial bone, transverse cross-sections were made perpendicular to the long axis, starting from the point where the fibula grows into it. From the femoral bone, a longitudinal section of the distal epiphysis was made in the median part and plane. The bone sections were ground on tarnished glass.

Histomorphometrical measurements were made using an Optiphot 2 microscope connected to a RGB camera and a personal computer (software: Lucia G 4.51, Laboratory Imaging), with final magnifications of $200\times$ and $500\times$. In the unstained preparations, the distance between the tetracycline stripes was measured on the periosteum side and on the marrow cavity side (periosteal and endosteal transverse growth) in UV light. In the stained preparations of the transverse cross-section of the tibia, the width of the endosteal and periosteal osteoid were determined. In the longitudinal preparations from the femur, the width of the epiphyseal cartilage and the width of trabeculae in the epiphysis and metaphysis were measured.

The transverse cross-sectional area of the cortical bone of the diaphysis and that of the marrow cavity in the tibia were measured in the stained preparations, with the use of a lanameter (magnification: $50\times$).

To determine the content of mineral substances in bones, the left tibia, left femur and L-4 vertebra were mineralized at a temperature of 640°C for 48 hours and weighed. The mineralized bones were dissolved in 6 M HCl, and the calcium and phosphorus content in the bone mineral was then assayed using colorimetric methods (using kits produced by Pointe Scientific, Inc.).

The mechanical properties of the whole femur and femoral neck were measured as previously described [31], using a set constructed at the Department of Pharmacology, Medical University of Silesia, in cooperation with Hottinger Baldwin Messtechnik GmbH.

The mechanical properties of whole left femurs supported on their epiphyses were studied using a three-point bending test. The load-deformation curves obtained for each bone, which represent the relationship between the load applied to the bone and the deformation in response to the load, were analyzed. The extrinsic stiffness of bone (the slope of the load-deformation curve), the ultimate and breaking loads (maximum load sustained by the bone and the load at fracture, respectively) and the deformation caused by the ultimate and breaking loads were determined. The mechanical properties of the femoral neck were studied using a compression test. The load was applied to the head of the femur along the long axis of the bone. The load causing the fracture of the femoral neck was determined.

The results are given in tables as the arithmetic mean values ($\pm$ SEM). The results obtained in the ovariectomized control rats (group V) were compared with those of the sham-operated control rats (group I) using the Student's *t*-test. One-way ANOVA followed by Dunnett's *post-hoc* test were used for statistical evaluation of the differences between results obtained after administration of thalidomide with those of the appropriate control rats (groups I or V).

Results

Effects of thalidomide on non-ovariectomized rats

In sham-operated rats receiving thalidomide at doses of 15, 30 or 60 mg/kg, *po*, no statistically significant differences in the studied parameters of the skeletal system were observed in comparison with the sham-operated control rats (data not shown).

The only statistically significant changes observed after administration of thalidomide were increased body mass gain and liver mass in rats receiving the drug at the highest dose. After 4 weeks of thalidomide administration at a dose of 60 mg/kg, *po*, there was a 37.5% increase in body mass gain and a 27.5% increase in liver mass compared to sham-operated control rats.

Effects of thalidomide on ovariectomized rats

Ovariectomy in the control rats (group V), as compared with the control sham-operated rats (group I), led to the following consequences: a 73.2% increase in body mass gain, a decrease in the uterus mass by 72.2% and a 58.0% increase in the thymus mass (Tab. 1). Thalidomide administered to ovariectomized rats at a dose of 60 mg/kg, *po* increased the body mass gain by 24.3%, and statistically insignificantly increased liver mass by 17.5%, when compared to the ovariectomized controls (Tab. 1).

No significant differences were observed in the mass of the femur, tibia and L-4 vertebra, the length of the femur and tibia or the diameter of their diaphyses and epiphyses between the ovariectomized controls and the thalidomide-treated ovariectomized rats (data not shown).

Histomorphometric examinations of cancellous bone were performed through measurement of trabecular width in the femoral epiphysis and metaphysis. The ovariectomized controls displayed a decrease in the trabecular width in the epiphysis (28.6%) and in the metaphysis (17.6%), when compared to the sham-operated control rats. Thalidomide treatment of ovariectomized rats at a dose of 15 mg/kg, *po* caused a 9.2% increase in trabecular width in the epiphysis, and a 9.1% increase in the metaphysis, compared to the ovariectomized controls. Thalidomide, at a dose of 60 mg/kg, *po*, decreased the trabecular width in the femoral epiphysis by 15.5% in ovariectomized rats, as compared to the results obtained in the ovariectomized controls (Tab. 2).

No significant differences in the width of the femoral epiphyseal cartilage were observed after administration of thalidomide to ovariectomized rats, compared to the ovariectomized controls (Tab. 2).

Histomorphometric examinations of the compact bone demonstrated a 17.9% increase in the width of the tibial osteoid on the side of periosteum and a 30.9% increase on the side of the endosteum in the

Tab. 1. Effects of thalidomide on the body mass and mass of several organs in ovariectomized (OVX) rats

Body mass and organ mass		SHAM	OVX	OVX + THALIDOMIDE		
				15 mg/kg, <i>po</i>	30 mg/kg, <i>po</i>	60 mg/kg, <i>po</i>
Body mass [g]	Initial	211.65 ± 2.63	207.01 ± 2.90	205.83 ± 3.10	207.07 ± 2.94	205.29 ± 2.01
	After 4 weeks	238.74 ± 3.31	253.92 ± 3.04 ^{SS}	253.18 ± 2.16	257.17 ± 2.49	263.59 ± 3.00 ^A
Increase after 4 weeks [g]		27.09 ± 2.78	46.91 ± 4.44 ^{SS}	47.36 ± 2.88	50.10 ± 1.98	58.30 ± 2.63 ^A
Organ mass [g]	Uterus	0.323 ± 0.042	0.090 ± 0.002 ^{SS}	0.090 ± 0.011	0.089 ± 0.008	0.097 ± 0.012
	Liver	7.31 ± 0.44	8.31 ± 0.45	8.70 ± 0.39	9.03 ± 0.38	9.76 ± 0.42 ^A
	Spleen	0.346 ± 0.049	0.358 ± 0.035	0.353 ± 0.026	0.348 ± 0.021	0.381 ± 0.026
	Thymus	0.328 ± 0.008	0.519 ± 0.041 ^{SS}	0.526 ± 0.041	0.502 ± 0.028	0.496 ± 0.026

Results are presented as the means ± SEM (n = 7). The results obtained in the OVX control rats were compared with those of the sham-operated (SHAM) control rats using Student's *t*-test. One-way ANOVA followed by Dunnett's *post-hoc* test were used to evaluate the statistical significance of the differences between results obtained after administration of thalidomide in OVX rats with those of the OVX control rats. ^{SS} p < 0.01 – statistically significant differences in comparison with the SHAM control rats. ^A p < 0.05 – statistically significant differences in comparison with the OVX control rats

Tab. 2. Effects of thalidomide on bone histomorphometric parameters in ovariectomized (OVX) rats

Bone histomorphometric parameters		SHAM	OVX	OVX + THALIDOMIDE		
				15 mg/kg, <i>po</i>	30 mg/kg, <i>po</i>	60 mg/kg, <i>po</i>
FEMUR						
Width of trabeculae [μm]	Epiphysis	75.33 ± 3.15	53.81 ± 1.84 ^{SS}	58.78 ± 0.90 ^A	55.40 ± 1.61	45.46 ± 2.81 ^A
	Metaphysis	45.16 ± 1.60	37.22 ± 1.29 ^{SS}	40.61 ± 1.57	37.81 ± 1.19	33.83 ± 2.43
Width of epiphyseal cartilage [μm]		94.40 ± 2.49	102.78 ± 3.78	98.19 ± 2.98	99.06 ± 2.09	101.48 ± 1.67
TIBIA						
Diaphysis transverse growth [μm]	Periosteal	45.62 ± 1.17	48.20 ± 5.45	46.86 ± 2.72	46.33 ± 2.61	36.85 ± 4.12
	Endosteal	19.69 ± 0.72	18.16 ± 2.69	16.65 ± 1.32	17.12 ± 1.20	16.39 ± 0.90
Width of osteoid [μm]	Periosteal	17.95 ± 0.45	21.17 ± 0.49 ^{SS}	16.03 ± 2.21 ^A	21.65 ± 0.97	18.59 ± 0.77 ^A
	Endosteal	8.24 ± 0.47	10.78 ± 0.70 ^{SS}	7.98 ± 0.96 ^A	10.59 ± 0.70	8.24 ± 0.74 ^A
Transverse cross-sectional area [mm ²]	Cortical bone	3.050 ± 0.047	2.985 ± 0.073	3.211 ± 0.219	3.102 ± 0.150	2.743 ± 0.167
	Marrow cavity	0.834 ± 0.029	0.989 ± 0.055 ^S	0.856 ± 0.023 ^A	0.982 ± 0.030	1.321 ± 0.133 ^A
	Diaphysis	3.883 ± 0.072	3.975 ± 0.091	4.067 ± 0.203	4.084 ± 0.149	4.064 ± 0.199
	Ratio [#]	0.214 ± 0.004	0.249 ± 0.011 ^{SS}	0.215 ± 0.018	0.243 ± 0.012	0.324 ± 0.028 ^A

[#] – the transverse cross-sectional area of the marrow cavity/transverse cross-sectional area of the diaphysis ratio. Results are presented as the means ± SEM (n = 7). The results obtained in the OVX control rats were compared with those of the sham-operated (SHAM) control rats using Student's *t*-test. One-way ANOVA followed by Dunnett's *post-hoc* test were used to evaluate the statistical significance of the differences between results obtained after administration of thalidomide in OVX rats with those of the OVX control rats. ^S p < 0.05, ^{SS} p < 0.01 – statistically significant differences in comparison with the SHAM control rats. ^A p < 0.05 – statistically significant differences in comparison with the OVX control rats

Tab. 3. Effects of thalidomide on bone mineral, calcium and phosphorus content in ovariectomized (OVX) rats

Parameters	SHAM	OVX	OVX + THALIDOMIDE		
			15 mg/kg, <i>po</i>	30 mg/kg, <i>po</i>	60 mg/kg, <i>po</i>
FEMUR					
Mineral content [mg/100 mg bone mass]	43.18 ± 1.27	38.17 ± 1.09 ^{SS}	38.52 ± 1.85	38.07 ± 1.12	38.43 ± 1.21
Calcium content [mg/100 mg mineral substances]	38.36 ± 1.69	32.84 ± 0.92 ^S	32.52 ± 0.76	34.53 ± 0.99	32.31 ± 1.12
Phosphorus content [mg/100 mg mineral substances]	35.12 ± 1.17	32.75 ± 1.94	32.83 ± 0.94	34.63 ± 1.31	31.39 ± 4.71
TIBIA					
Mineral content [mg/100 mg bone mass]	43.83 ± 1.99	38.64 ± 1.15 ^{SS}	40.93 ± 2.14	40.95 ± 2.33	38.11 ± 1.27
Calcium content [mg/100 mg mineral substances]	39.31 ± 1.22	35.29 ± 1.03 ^S	33.40 ± 1.76	33.89 ± 3.04	33.19 ± 1.50
Phosphorus content [mg/100 mg mineral substances]	36.04 ± 1.33	32.27 ± 1.15	30.05 ± 2.54	32.79 ± 2.34	31.79 ± 1.93
L-4 VERTEBRA					
Mineral content [mg/100 mg bone mass]	36.45 ± 0.80	32.43 ± 1.55 ^{SS}	33.18 ± 1.53	32.97 ± 1.14	33.13 ± 1.39
Calcium content [mg/100 mg mineral substances]	40.42 ± 0.96	33.29 ± 2.79 ^S	32.87 ± 2.91	32.74 ± 2.07	30.05 ± 1.83
Phosphorus content [mg/100 mg mineral substances]	37.19 ± 0.73	33.85 ± 1.72	34.34 ± 2.83	35.81 ± 2.77	31.59 ± 3.06

Results are presented as the means ± SEM (n = 7). The results obtained in the OVX control rats were compared with those of the sham-operated (SHAM) control rats using Student's *t*-test. One-way ANOVA followed by Dunnett's *post-hoc* test were used to evaluate the statistical significance of the differences between results obtained after administration of thalidomide in OVX rats with those of the OVX control rats. ^S p < 0.05, ^{SS} p < 0.01 – statistically significant differences in comparison with the SHAM control rats

Tab. 4. Effects of thalidomide on the mechanical properties of bones from ovariectomized (OVX) rats

Parameters		SHAM	OVX	OVX + THALIDOMIDE		
				15 mg/kg, <i>po</i>	30 mg/kg, <i>po</i>	60 mg/kg, <i>po</i>
FEMORAL DIAPHYSIS						
Load [N]	Ultimate	83.92 ± 1.93	77.92 ± 2.72	79.43 ± 1.43	78.76 ± 1.14	77.20 ± 1.03
	Breaking	77.08 ± 1.74	55.48 ± 4.95 ^{SS}	53.94 ± 3.90	57.52 ± 2.20	56.82 ± 2.09
Deformation [mm]	At ultimate load	0.398 ± 0.006	0.453 ± 0.025	0.435 ± 0.009	0.450 ± 0.009	0.436 ± 0.014
	At breaking load	0.478 ± 0.012	0.517 ± 0.016	0.493 ± 0.010	0.498 ± 0.011	0.493 ± 0.019
Extrinsic stiffness [N/mm]		215.71 ± 26.4	157.43 ± 5.69 ^{SSS}	168.14 ± 16.01	155.29 ± 24.13	155.71 ± 14.56
FEMORAL NECK						
Load at fracture [N]		75.78 ± 1.21	57.24 ± 2.41 ^{SSS}	65.87 ± 2.68 ^A	56.73 ± 2.29	57.52 ± 2.25

Results are presented as the means ± SEM (n = 7). The results obtained in the OVX control rats were compared with those of the sham-operated (SHAM) control rats using Student's *t*-test. One-way ANOVA followed by Dunnett's *post-hoc* test were used to evaluate the statistical significance of the differences between results obtained after administration of thalidomide in OVX rats with those of the OVX control rats. ^{SS} p < 0.01, ^{SSS} p < 0.001 – statistically significant differences in comparison with the SHAM control rats. ^A p < 0.05 – statistically significant differences in comparison with the OVX control rats

ovariectomized control rats, when compared to the sham-operated control rats. Thalidomide at a dose of 15 mg/kg, *po* decreased the width of the periosteal and endosteal osteoid by 24.3% and 26.0%, respectively, in ovariectomized rats compared with the ovariectomized controls. The ovariectomized rats that were treated with 60 mg/kg, *po* thalidomide displayed a 12.2% decrease in the osteoid width on the side of periosteum, and a 23.5% decrease on the side of endosteum compared to the ovariectomized controls (Tab. 2).

An 18.7% increase in the tibial marrow cavity transverse cross-sectional area was observed in the ovariectomized controls in comparison with the sham-operated control rats. Also, the marrow cavity transverse cross-sectional area/diaphysis transverse cross-sectional area ratio was 16.0% larger than in the controls (Tab. 2). Thalidomide administered to the ovariectomized rats at a dose of 15 mg/kg, *po* decreased the transverse cross-sectional area of the marrow cavity by 13.5% compared to the ovariectomized control group. Also, the marrow cavity transverse cross-sectional area/diaphysis transverse cross-sectional area ratio was smaller by 9.7% in the group receiving 15 mg/kg, *po* thalidomide, although this change was not statistically significant. The transverse cross-sectional area of the marrow cavity and the marrow cavity transverse cross-sectional area/diaphysis transverse cross-sectional area ratio were increased by 33.4% and 30.4%, respectively, in the ovariectomized rats receiving 60 mg/kg, *po* thalidomide, compared to the ovariectomized controls (Tab. 2).

No significant differences were observed in regard to the transverse growth of the tibia in the ovariectomized controls and ovariectomized rats that were treated with 15 and 30 mg/kg, *po* thalidomide. Thalidomide at a dose of 60 mg/kg, *po* decreased transverse growth on the side of periosteum by 23.5% in ovariectomized rats, as compared to the ovariectomized control rats, but this result was not statistically significant (Tab. 2).

In the ovariectomized control rats, the mineral content was reduced in the femur by 11.5%, in the tibia by 11.9% and in the L-4 vertebra by 10.9%, when compared to the sham-operated control rats. The mineral content of the tested bones from groups receiving thalidomide was similar to that of the ovariectomized controls (Tab. 3).

The calcium content of bones from ovariectomized controls was reduced in the femur by 14.4%, in the tibia by 10.2% and in the L-4 vertebra by 17.6% compared to the sham-operated control rats (Tab. 3). The administration of thalidomide did not affect the calcium content in the bones of ovariectomized rats.

The phosphorus content in the femur, tibia and L-4 vertebra of the ovariectomized control rats was statistically insignificantly reduced by 6.8%, 10.5% and 9.0%, respectively, compared to the sham-operated control rats (Tab. 3). The administration of thalidomide did not affect the phosphorus content in the bones of ovariectomized rats.

In the ovariectomized control rats, the ultimate load endured by the whole femur was similar to that of the sham-operated control rats, but the breaking load was decreased by 28.0%. In the ovariectomized rats, the femoral deformation caused by the ultimate and breaking loads was insignificantly larger by 13.8% and 8.1%, respectively, and the extrinsic stiffness of the femur was significantly smaller (27.0%) than in the sham-operated control rats. The breaking load of the neck in the ovariectomized rats was smaller by 24.5% (Tab. 4).

In ovariectomized rats receiving thalidomide at all doses, the ultimate load, the breaking load and the deformation exhibited by the femoral diaphysis (both at the ultimate load and at the moment of fracture) were similar to those observed for the ovariectomized control rats. Thalidomide administered at a dose of 15 mg/kg, *po* to ovariectomized rats increased the breaking load of the femoral neck by 15.1% and statistically insignificantly increased the extrinsic stiffness of the whole femur by 6.8% compared to the ovariectomized controls (Tab. 4).

Discussion

The favorable effects of thalidomide in the treatment of multiple myeloma are caused by the inhibition of cytokine production, and the subsequent inhibition of osteoclastogenesis [17]. This mechanism suggests the possibility that thalidomide could affect the rat skeletal system *in vivo*. The possible inhibition of bone resorption by thalidomide could have favorable effects on the development of bone changes caused by estrogen deficiency.

In the present work, thalidomide was administered once daily, at doses of 15, 30 or 60 mg/kg. The doses chosen for the experiments in rats took into account the doses normally used in humans. In clinical trials with patients and volunteers, thalidomide is used orally, usually at doses ranging from 100 to 400 mg daily [23, 25], which is equivalent to about 1.5 mg/kg to 6.0 mg/kg of adult male body mass. Because the systemic metabolism of rats is more intense than that of humans, a 10× correction factor, commonly used in pharmacological studies, was used. Also, similar doses of thalidomide were successfully used by others in studies with experimental animals [34, 42, 45].

Thalidomide administered for 4 weeks at doses of 15, 30 or 60 mg/kg, *po* did not affect the skeletal system of sham-operated female rats. The only changes observed after administration of thalidomide were the increased body mass gain and increased liver mass in rats receiving thalidomide at a dose of 60 mg/kg, *po*. These effects were also observed in ovariectomized rats, and have not been previously reported in the literature. Although the main pathway for thalidomide metabolism is the spontaneous, nonenzymatic pH-dependent hydrolysis that occurs in body fluids [7], thalidomide may also, to a lesser extent, undergo hydroxylation by cytochrome P450 isoenzymes in liver microsomes both in humans and rats [3]. Because the exact mode of thalidomide metabolism in humans and rats is not clear, it seems possible that the increase in the liver mass in rats receiving the drug at a dose of 60 mg/kg, *po* may be the result of effects on the activities of liver microsomal enzymes. The changes in the liver mass may also be due to liver damage. This problem requires further investigation.

The effects of thalidomide on the rat skeletal system were demonstrated only in ovariectomized rats. The model used in this study, in which bilateral ovariectomy induced osteoporosis in rats, may reflect disturbances characteristic of osteoporosis, which develops in women after menopause as a result of estrogen deficiency [6]. This animal model of osteoporosis has been commonly used to examine the effects of different drugs on the skeletal system [10, 43, 46]. Estrogen deficiency leads to an increase in the bone remodeling rate (both bone resorption and formation). Experimental data reveal that these changes may be due to the increased release of cytokines that increase bone resorption (IL-1, IL-6, TNF- α), and are normally controlled by estrogens [28, 33]. The changes in remodeling rate may lead to the devel-

opment of osteoporosis in postmenopausal women. Similarly, increased bone marrow cell secretion of IL-1, IL-6 and TNF- α was observed in ovariectomized rats [18, 21].

In the present study, bilateral ovariectomy in sexually mature female rats caused a statistically significant increase in body weight and thymus mass, as well as a significant decrease in the uterus mass. The existence of estrogen deficiency 4 weeks after the bilateral ovariectomy also caused profound changes in the osseous system. The observed changes are in accord with our earlier observations [8, 14, 15], as well as with observations made by others [16]. Histomorphometric measurements performed in our study indicated that the process of osseous tissue remodeling was disturbed both in the cortical and trabecular bone. In the cortical bone of the tibia, the process of resorption was intensified, as evidenced by the increase in the marrow cavity transverse cross-sectional area and the increased ratio of the marrow cavity transverse cross-sectional area to the diaphysis transverse cross-sectional area. Also, bone formation was intensified, as evidenced by the increase in the osteoid width. Bone resorption was increased in the trabecular bone of the femur, and we observed a statistically significant decrease in the trabecular width in the epiphysis and metaphysis. The ovariectomized control rats exhibited a diminished mineral content, calcium content and phosphorus content in the examined bones, which could be due to increased bone resorption and/or the impairment of bone mineralization. The osteoporotic changes in the ovariectomized control rats caused statistically significant worsening of the investigated mechanical parameters. In the whole femur measurements, when the load was applied to the diaphysis consisting of cortical bone, the extrinsic stiffness and the breaking load were decreased compared to controls. In the femoral neck, which consists mainly of cancellous bone, the load at fracture was decreased compared to controls.

The effects of thalidomide on the osseous tissue of ovariectomized rats were dependent upon the dose. At the lowest dose, thalidomide treatment favorably affected the skeletal system, whereas at the highest dose, it caused intensification of the osteoporotic changes.

Thalidomide, given at a dose of 15 mg/kg, *po*, significantly counteracted some of the histomorphometric changes induced by estrogen deficiency in the tibia (in the width of the periosteal and endosteal osteoid, the marrow cavity transverse cross-sectional area and

its ratio to the transverse cross-sectional area of the diaphysis) and in the femur (in the width of epiphyseal and metaphyseal trabeculae). These results indicate that thalidomide inhibits the increased bone resorption and formation induced by estrogen deficiency. Thalidomide also improved bone mechanical properties. Treatment increased the load at fracture of the femoral neck and the extrinsic stiffness of the whole femur. The improvement could be due to decreased bone resorption. Thalidomide did not affect bone macrometric parameters or the mineral, calcium and phosphorus content of bones from the ovariectomized rats. Taken together, the results presented herein suggest that thalidomide, at a dose of 15 mg/kg, *po*, alleviated the changes in histomorphometric and mechanical parameters induced by estrogen deficiency in ovariectomized rats; however, it did not repair the disordered mineralization.

The mechanism by which the lowest dose of thalidomide produced favorable effects in the skeletal system of ovariectomized rats might be hypothesized. Currently, the effect of thalidomide on the synthesis, release and activity of TNF- α , IL-1 and IL-6 has been confirmed, and is considered the basis of the drug's anti-inflammatory, immunomodulatory and antineoplastic properties [5, 36, 37]. *In vitro* experiments demonstrated that thalidomide is a potent inhibitor of TNF- α synthesis in monocytes [36] and macrophages [20]. Thalidomide also inhibits the production of IL-6 and TNF- α in peripheral blood mononuclear cell culture [35], and decreases TNF- α synthesis in CD4⁺ and CD8⁺ T cells [22]. Thalidomide abolishes the endogenous overproduction of TNF- α in patients suffering from various different diseases [1, 25–27, 32, 47]. Clinical observations confirmed that the anti-inflammatory properties of thalidomide utilized for the treatment of rheumatoid arthritis, inflammatory bowel disease and Crohn's disease are due to the inhibitory effect on TNF- α and/or IL-1 [4, 30, 37]. Since estrogen deficiency-related osteoporosis is presumably linked to the increased release of proresorptive cytokines (IL-1, IL-6 and TNF- α) [18, 21, 28, 33], one may presume that the alleviation of bone resorption and improvement of bone mechanical properties observed here in ovariectomized rats receiving 15 mg/kg, *po* thalidomide, were the result of its effect on those cytokines. In our *in vitro* studies on LPS-stimulated murine osteoblasts, thalidomide decreased the expression of TNF- α and IL-6 mRNA [12]. However, Lin et al. [20] did not observe the re-

duction of TNF- α and IL-6 synthesis by thalidomide in osteoblasts stimulated by titanium particles. Our experiments, however, do not provide an explanation for the lack of any thalidomide effect on the disordered bone mineralization caused by estrogen deficiency.

Administration of 30 mg/kg, *po* thalidomide to ovariectomized rats did not markedly affect the examined bone parameters. Thalidomide treatment at the highest dose (60 mg/kg, *po*) exerted a destructive effect on the skeletal system of ovariectomized rats; such an effect was not observed in sham-operated rats. In comparison with the ovariectomized control rats, bone resorption processes were intensified. Specifically, we observed increases in the transverse cross-sectional area of the marrow cavity and the ratio of the transverse cross-sectional area of the marrow cavity to the transverse cross-sectional area of the diaphysis in the tibia. We also observed decreases in the width of epiphyseal and metaphyseal trabeculae in the femur. Bone formation processes were inhibited, as evidenced by a reduction in the width of the periosteal and endosteal osteoid and in the periosteal transverse growth. The increased bone resorption observed after administration of thalidomide at a dose of 60 mg/kg, *po* may be due to increased osteoclastogenesis. In our *in vitro* study, thalidomide intensified osteoclastogenesis in neonatal murine bone marrow cell culture in a concentration-dependent manner. At a concentration of 25 μ g/ml, thalidomide significantly increased the number and the diameter of osteoclasts [13]. However, the experiments of Anderson et al. [2] with human bone marrow cells demonstrated the inhibition of osteoclastogenesis by thalidomide at concentrations of 1–100 μ M.

In summary, thalidomide did not affect the skeletal system of non-ovariectomized female rats. The effects of thalidomide on the skeletal system of ovariectomized rats depended on the dose used. At a dose of 15 mg/kg, *po*, thalidomide counteracted some osteoporotic changes induced by estrogen deficiency. At a dose of 60 mg/kg, *po*, thalidomide intensified the destructive effects of estrogen deficiency on the rat skeletal system. The results of the present study indicate the possibility of unfavorable side effects of thalidomide therapy on the skeletal system of patients. On the other hand, there is also a possibility that thalidomide may be efficacious in some metabolic bone diseases, including postmenopausal osteoporosis.

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