Raloxifene is a selective estrogen receptor modulator (SERM), having an agonist-antagonist action towards estrogen receptors. The antagonist action of raloxifene is revealed in the uterus and breast. The agonist action of raloxifene is visible in bones and the cardiovascular system.

The assessment of raloxifene action on the osseous tissue is based on numerous experimental studies carried out on animals as well as on numerous clinical tests. It has been demonstrated that the administration of raloxifene in female rats prevented ovariectomy-induced bone loss (1). Clinical tests performed on postmenopausal women confirmed positive effect of raloxifene on the osseous tissue. The effects of raloxifene observed in postmenopausal women include: significant reduction in markers of bone formation (serum osteocalcin, bone-specific alkaline phosphatase, urinary excretion of type I collagen C-telopeptide) (2). Additionally, tests were carried out for postmenopausal women with bone fractures, who displayed increased osseous tissue density as a result of raloxifene administration (3, 4). Therefore, the drug has been used in prevention and treatment of osteoporosis in postmenopausal women.

There have also been isolated scientific reports suggesting the possibility of the drug having anti-osteoporosis effect in men who display altered estrogen concentration in blood (5–8). However, the possibility of using raloxifene in osseous tissue disorders in men has not been unambiguously documented.

Tacrolimus, which belongs to immunosuppressive drugs, prevents and stops the already-initiated process of transplant rejection. Thus, it has been administered to patients with allogenic organ transplants.
Tacrolimus has a controversial effect on the osseous tissue. A majority of researchers claim it has a destructive effect on bone processes which lead to osteoporosis (9, 10). However, some literature data, based on test results obtained in other experimental systems, suggest a positive effect of the drug on bones (11).

Therefore, it seems purposeful to examine effects of both raloxifene and tacrolimus on the osseous tissue in male rats. We decided to make an initial assessment of the effects of raloxifene, tacrolimus and concurrent raloxifene-tacrolimus administration on histomorphometric parameters of bones in male rats in order to investigate the influence of the drugs on the osseous tissue.

EXPERIMENTAL

The experiments were carried out on mature male Wistar rats (275–290 g) from the Central Animal Farm of the Silesian Medical University. The permission for the animal tests and experiments has been given by the Local Ethics Commission, Katowice.

The animals were divided into six groups, 7 animals each: I (C) – control rats; II (R) – rats which were administered raloxifene (5 mg/kg po daily; Evista); III (T/0.3) – rats which were administered tacrolimus (0.3 mg/kg po daily; Prograf®); IV (T/0.6) – rats which were administered tacrolimus (0.6 mg/kg po daily); V (R + T/0.3) – rats which were administered raloxifene (5 mg/kg po daily) and tacrolimus (0.3 mg/kg po daily); VI (R + T/0.6) – rats which were administered raloxifene (5 mg/kg po daily) and tacrolimus (0.6 mg/kg po daily). The drugs were administered for 4 weeks.

Twenty four hours prior to the first administration and on the last day of administration of the drugs, the animals were given tetracycline hydrochloride (20 mg/kg ip) in order to mark the calcification front. Tetracycline was a histomorphometric fluorescence marker (12).

After 4 weeks of drugs administration, all the animals were sacrificed. The right and left femoral and tibial bones and L-4 vertebra were isolated. After isolation and freeing from muscular tissue the bones were weighed.

Macrometric parameters (length and diameter of the diaphysis in the mid-length) in the isolated bones were determined. A slide caliper was used for the macrometric measurements.

The femoral and tibial bones were used to prepare histological specimens. From the tibial bone, transverse cross-sections were made, perpendicular-ly to the long axis, starting from the point where fibula grows into it. Three tibial slices were obtained by cutting. From the femoral bone, a longitudinal section of the distal epiphysis was made, in the medial part and plane. The sections were ground on the tarnished glass. The first preparation from the tibia was remained unstained. The rest of the preparations (2nd and 3rd tibial cross-section slices together with the longitudinal section slice of the femoral distal epiphysis) were stained, undecalcified using the Tripp and MacKay method (13). Staining times were subjected to the authors’ own modification.

Histomorphometrical measurements were made using Optiphot 2 (Nikon) microscope, connected by RGB camera (Cohu) with a personal computer (program Lucia G 4.51), with final magnifications of 200 and 500 times.

In the unstained preparation, the distance between the tetracycline stripes were measured, on the periosteum side and on the marrow cavity side (periosteal and endosteal transverse growth). Determinations of transverse growth of the tibia were done in the UV light on unstained preparation whereas determinations of other histomorphometrical parameters were done in the visible light.

In the stained preparation of the transverse cross-section of the tibia, the width of the endosteal and periosteal osteoid were determined. In the longitudinal preparation from the femur, the width of epiphysseal cartilage and the width of trabeculae in the epiphysis and metaphysis were measured.

The transverse cross-section area of the cortical part and of the marrow cavity in the tibia were measured in the stained preparation, with the use of a lanameter with magnification 50 times.

Results are presented as the mean ± SEM. One-way ANOVA followed by post-hoc Duncan’s multiple-range test was used for estimation of statistical signification. Results obtained in all experimental groups were compared with the control group. Groups R+T/0.3 and R+T/0.6 also compared with group R and groups T/0.3 or T.0.6, respectively.

RESULTS

The determinations of the effects of raloxifene, tacrolimus as well as raloxifene and tacrolimus administered concurrently on macrometric parameters of bones in rats

The initial body mass in the controls (group C) was 275.72 ± 5.32 g. After four weeks of the experiment, the body mass in this group was 325.43 ± 7.07g, which meant an increase by 49.71 ± 3.38g.
Rats which received raloxifene for the period of four weeks (group R) displayed a statistically significantly smaller body mass increase ($p < 0.001$), i.e. by 51.28% when compared to the controls.

Animals which received tacrolimus at a dose of 0.3 mg/kg (group T/0.3) or 0.6 mg/kg (group T/0.6) for four weeks, displayed an increase in body mass similar to group C.

After four weeks of concurrent administration of raloxifene and tacrolimus at a dose of 0.3 mg/kg (group R+T/0.3), the increase of the body mass was statistically significantly smaller ($p < 0.001$) when compared to group C (by 59.92%). Rats administered raloxifene and tacrolimus at a dose of 0.6 mg/kg concurrently for four weeks (group R+T/0.6) displayed a statistically significantly smaller body mass increase ($p < 0.001$) compared to group C (by 59.90%).

The masses of femur, tibia and L-4 vertebrae in group C were: 0.89 ± 0.01 g, 0.63 ± 0.01 g and 0.25 ± 0.01 g, respectively. In groups: R, T/0.3, T/0.6, R+T/0.3 or R+T/0.6, the mass of the femur, tibia, L-4 vertebrae was similar to the results obtained for the controls.

The length of femur in group C was 36.78 ± 0.18 mm, whereas the length of tibia was 40.78 ± 0.07 mm. The administration of the examined drugs in groups: R, T/0.3, T/0.6, R+T/0.3 or R+T/0.6, did not alter the femoral or tibial length when compared to group C.

In group C the diameter of the femoral diaphysis measured in frontal plane half-length of the bone was 3.89 ± 0.16 mm, the diameter measured in medial plane was 3.30 ± 0.06 mm. In this group the diameter of the tibia determined half-length of the bone in frontal plane was 3.43 ± 0.10 mm and in medial plane: 2.44 ± 0.02 mm. In case of groups: R, T/0.3, T/0.6, R+T/0.3 or R+T/0.6, the diameter of the tibial and femoral diaphysis was similar to those of group C (data not shown).

The determinations of the effects of raloxifene, tacrolimus as well as raloxifene and tacrolimus administered concurrently on histomorphometric parameters of bones in rats

The results of raloxifene and tacrolimus effects on histomorphometric parameters are presented in Table 1.

The transverse cross section area of the cortical bone and marrow cavity in the tibia in group R were similar to those of group C.

In case of group T/0.3 a statistical increase of both: the transverse cross section area of the cortical bone in the tibia by 22.94% ($p < 0.001$), as well as the transverse cross section area of the marrow cavity in the tibia by 25.36% ($p < 0.01$) were observed when compared to group C. In group T/0.6, the transverse cross section areas of the cortex and marrow cavity were similar to the results obtained in group C.

Concurrent administration of raloxifene and tacrolimus in group R+T/0.3 resulted in a statistically significant increase in the transverse cross-section ($p < 0.001$) of the cortical bone, by 30.27%, and statistically significant increase ($p < 0.05$) of the cross section area of marrow cavity by 17.48% when referred to group C.

The group R+T/0.6 displayed a statistically significant increase ($p < 0.01$) of the transverse cross-section area of the cortical bone, by 15.92%, when compared to group C. This result was also statistically significant in comparison with group R ($p < 0.001$) and group T/0.6 ($p < 0.01$).

The rats in group R demonstrated a periosteal transverse growth of the tibia similar to the results obtained in group C, whereas the endosteal transverse growth of the same bone was 12.56% higher than in controls.

Administration of tacrolimus in groups T/0.3 and T/0.6 resulted in an increase of endosteal transverse growth of the tibia by 10.53% and 14.66%, respectively, when compared to the results obtained in group C.

Animals which were given raloxifene and tacrolimus concurrently in groups R+T/0.3 and R+T/0.6, demonstrated increased endosteal tibial transverse growth by 18.32% and 20.56%, respectively, when compared to the controls. Tacrolimus and raloxifene with tacrolimus did not affect the periosteal transverse growth in comparison with the control group.

In case of group R, the periosteal and endosteal osteoid width was bigger by 9.65% and 18.98%, respectively, compared to the results obtained in group C.

The administration of tacrolimus in groups T/0.3 and T/0.6 caused an increased periosteal osteoid by 12.28% ($p < 0.05$) and 15.61% ($p < 0.05$), respectively, and an increase of endosteal osteoid by 37.89% and 19.71%, respectively, when compared to group C.

Rats in groups R+T/0.3 and R+T/0.6 displayed periosteal osteoid increase of 11.0% ($p < 0.05$) and 15.0% ($p < 0.05$), respectively, whereas endosteal osteoid was increased by 22.44% and 26.74%, respectively, compared to the controls.

In group R, the width of trabeculae, both in the epiphysis and metaphysis of the femur, were similar to the controls.
The administration of tacrolimus to T/0.3 group resulted in an increased width of trabeculae in the metaphysis of the femur by 7.21% when compared to the controls, whereas the thickness of epiphyseal trabeculae remained unaffected. Similarly, group R+T/0.3 displayed an increase in metaphyseal trabeculae thickness by 5.75%.

The groups T/0.6 and R+T/0.6 demonstrated the width of femoral trabeculae, both in epiphysis and metaphysis, similar to the result obtained in the controls.

The femoral epiphyseal cartilage width of distant femur in group C was 35.88 ± 1.89 µm. No influence of the examined drugs on the epiphyseal cartilage width was observed in the femoral epiphysis (data not shown).

**DISCUSSION**

Raloxifene has an established position in the treatment of osteoporosis in post-menopausal women. Attempts have been made in recent years to use raloxifene in men at 60–120 mg (5–8). The tests showed anti-resorptive action of the drug, which suggests that raloxifene may be used in osteoporosis prophylaxis in men.

Raloxifene increases the secretion of osteoprotegerin, a potent inhibitor of bone resorption, and suppresses the production of the bone resorbing cytokine, IL-6. Regulation of those cytokines in the bone may be an important mechanism whereby raloxifene reduces bone resorption (14).

In our study we administered raloxifene at a dose of 5 mg/kg po per day for 4 weeks in order to investigate its influence on the osseous tissue in male rats, taking into account that in case of female rats, the drug was active against osteoporosis in the range of doses of 0.1 to 10 mg/kg po (1).

The administration of raloxifene in male rats resulted in a decreased body mass, while the mass and shape of the examined bones, i.e. the femur, the tibia and L-4 vertebrae, remained unaffected. Taking into account the histomorphometric parameters of the examined bones, the action of raloxifene was demonstrated in increased endosteal bone transverse growth and increased osteoid width, both periosteal and endosteal. This suggests that the use of raloxifene in male rats results in intensified bone formation (in particular the organic part of osseous tissue), which, however, was not demonstrated in the other examined parameters (transverse cross-section area and trabeculae thickness).

The effect of tacrolimus on the osseous tissue is controversial. Most scientific reports suggest that

<table>
<thead>
<tr>
<th>Examined parameters</th>
<th>C</th>
<th>R</th>
<th>T/0.3</th>
<th>T/0.6</th>
<th>R+T/0.3</th>
<th>R+T/0.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transverse cortex area in the femur [µm²]</td>
<td>3.77 ± 0.09</td>
<td>3.59 ± 0.08</td>
<td>3.63 ± 0.09</td>
<td>4.63 ± 0.09</td>
<td>4.91 ± 0.15</td>
<td>4.37 ± 0.28</td>
</tr>
<tr>
<td>Marrow cavity in the femur [µm²]</td>
<td>1.07 ± 0.06</td>
<td>1.12 ± 0.06</td>
<td>1.26 ± 0.02</td>
<td>1.26 ± 0.02</td>
<td>1.26 ± 0.02</td>
<td>1.26 ± 0.02</td>
</tr>
<tr>
<td>Transverse growth of the femur [µm]</td>
<td>73.69 ± 1.75</td>
<td>73.81 ± 2.62</td>
<td>73.90 ± 1.78</td>
<td>71.04 ± 1.61</td>
<td>70.43 ± 1.30</td>
<td>70.43 ± 1.30</td>
</tr>
<tr>
<td>Marrow cavity in the femur [µm³]</td>
<td>31.13 ± 0.49</td>
<td>35.16 ± 2.33</td>
<td>35.81 ± 0.91</td>
<td>36.04 ± 2.50</td>
<td>36.04 ± 2.50</td>
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</tr>
<tr>
<td>Width of osteoid in the femur [µm]</td>
<td>10.45 ± 0.51</td>
<td>12.43 ± 0.77</td>
<td>14.41 ± 1.11</td>
<td>12.51 ± 1.64</td>
<td>12.51 ± 1.64</td>
<td>12.51 ± 1.64</td>
</tr>
<tr>
<td>Width of trabeculae in the femur [µm]</td>
<td>60.35 ± 1.29</td>
<td>56.50 ± 1.34</td>
<td>45.19 ± 1.06</td>
<td>47.64 ± 0.45</td>
<td>45.19 ± 1.06</td>
<td>47.64 ± 0.45</td>
</tr>
</tbody>
</table>

ANOVA: p < 0.05, AA p < 0.01, AAA p < 0.001 ñ differences statistically significant compared to the results obtained for control rats (C), RRR p < 0.001 ñ differences statistically significant compared to the results obtained for rats of R group, TT p < 0.01 ñ differences statistically significant compared to the results obtained for rats of T/0.6 group.
Tacrolimus has a destructive effect on the osseous tissue, which may lead to osteoporosis.

The earlier examination demonstrated that the administration of tacrolimus in rats at doses of 1.5 mg/kg or at 3 mg/kg for 28 days resulted in a decreased bone mass and decreased mass of bone ash. This applied to both the cortical and trabecular bones. The tibial epiphyses displayed a decreased volume of trabecular bone (15).

Tacrolimus-induced osteopenia was also acknowledged in histomorphometric examinations and determinations of osseous tissue metabolism in rats, where tacrolimus was administered at 1 mg/kg and 3.2 mg/kg (16) as well as 4 mg/kg (17). The loss of trabecular bone mass was also revealed by Cvetkovic et al. (18) in histomorphometric tests carried out on rats administered tacrolimus.

It was reported that tacrolimus intensified bone turnover in rats, increasing the bone formation processes and resorption. This was demonstrated, among others, by Kirino et al. (19) on the basis of histomorphometric tests and determinations of bone resorption markers (osteocalcin, pyridinoline, deoxypyridinoline, parathyroid hormone) in rats receiving tacrolimus at 1 mg/kg for the period of 5 weeks. Cvetkovic et al. (18) observed intensified processes of bone formation and resorption in rats receiving tacrolimus at 5 mg/kg for 28 days (based on decreased calcium level and increased parathyroid hormone level).

There have also been reports proving that tacrolimus at lower doses may increase bone formation and even suggestions that the drug may be used in case of bone fractures. The administration of tacrolimus in rats with osseous cell implants on porous hydroxyapatite resulted in intensified bone formation (increased alkaline phosphatase activity and osteocalcin content) (11).

Not many examinations for tacrolimus effect on the osseous system were carried out on humans. The existing sparse reports prove that a loss of osseous tissue occurs, which leads to osteoporosis (9, 10).

Some researchers attempted to explain the mechanism of tacrolimus action on the osseous tissue. Tacrolimus (1 mg/kg ip) intensified bone induction in demineralized isogeneic and xenogeneic bone matrix in the rat and the increase in the tibia, both bone formation, and bone resorption, accompanied by a significant reduction in the relative trabecular area (20). The drug, administered to rats at 1 mg/kg im, intensified resorption of the trabecular bone and increased the number of osteoclasts in the points of ossification. The authors of the experiment suggest that the mechanism of tacrolimus-induced osteoporosis consisted in its effect on osteoclasts through intensified expression of RANKL mRNA, and accelerated differentiation and maturing of the cells (21).

In humans tacrolimus is administered intravenously or orally. Oral administration is usually at 0.15 to 0.3 mg/kg per day. In our study, the dose administered to rats was 0.3 and 0.6 mg/kg po daily for 4 weeks. No body mass changes were observed in rats receiving tacrolimus at both doses, when compared to the controls. The drug did not affect the mass of the femur, tibia and L-4 vertebrae, and it had no effect on the length and diameter of the femoral and tibial diaphyses.

Rats receiving tacrolimus at 0.3 mg/kg in our study displayed an increased tibial traverse growth, increased osteoid width, as well as increased traverse cross-section area of the tibial cortex and marrow cavity. The femur displayed increased width of metaphyseal trabeculae thickness. The results suggest that tacrolimus administered (0.3 mg/kg) to male rats caused intensified osseous tissue formation. The increased traverse cross section of the marrow cavity in tibia indicates that the resorption processes in the tissue were intensified. Therefore, the results of this study confirm the findings of earlier studies, which demonstrated that tacrolimus administration intensified both bone formation and resorption processes, which proves the general acceleration of osseous tissue metabolism (17, 19). This time, however, the intensified bone formation was dominant over the processes of resorption, thus no osteoporosis symptoms were observed.

Tacrolimus administered to male rats at 0.6 mg/kg also resulted in increased bone formation processes; this action, however, was much weaker than in case of rats receiving the drug at 0.3 mg/kg. And thus, tacrolimus at 0.6 mg/kg caused an increase bone traverse growth and increased osteoid width, with no influence on the traverse cross section of the tibial diaphysis and with unaffected width of femoral trabeculae.

In case of rats which were administered raloxifene and tacrolimus (at 0.3 mg/kg) concurrently, an increased traverse cross section of the cortex and marrow cavity was observed, as well as increased bone traverse growth, increased width of osteoid and increased width of trabeculae. The results are similar to those obtained in the groups given tacrolimus only (0.3 mg/kg). Hence, the present study did not demonstrate that raloxifene influenced the action of tacrolimus (0.3 mg/kg) on histomorphometric parameters of bones in male rats.
Similarly, in case of rats receiving raloxifene and tacrolimus at 0.6 mg/kg, raloxifene did not affect the action of tacrolimus on bone transverse growth and osteoid width. Simultaneously, it was observed that concurrent administration of raloxifene and tacrolimus at 0.6 mg/kg caused an increased transverse cross section of the cortical tibial diaphysis. However, this action was also observed in rats administered tacrolimus only, at 0.3 mg/kg.

CONCLUSION

The most valuable results obtained in the entire experimental system applied in this study seem to be the results obtained in the group administered tacrolimus at 0.3 mg/kg po, which are indicative of intensified bone remodeling processes with bone formation processes dominant.

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