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THE INFLUENCE OF IMMUNOSUPPRESSIVE TREATMENT DURING PREGNANCY ON FLUORIDE LEVELS IN THE HARD TISSUES OF FEMALE WISTAR RATS AND THEIR OFFSPRING

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ABSTRACT: In this study, we evaluated the influence of combination immunosuppressive therapy on fluoride levels in the teeth and bones of pregnant female Wistar rats and their offspring. The study was conducted on 40 female Wistar rats which were given the full or half dose of immunosuppressants in combinations commonly used in the treatment of kidney transplant recipients.The animals received medication by oral gavage 2 weeks prior to pregnancy and during 3 weeks of pregnancy. Our findings suggest that immunosuppressive medications, commonly used in humans after kidney transplantation, may affect F metabolism in bones and teeth, both in female rats exposed to this treatment and their offspring.

Keywords: Bone; Fluoride; Immunosuppressive therapy; Pregnancy; Teeth.

INTRODUCTION

Kidney transplantation is the treatment of choice in patients with end-stage renal disease (ESRD). It is linked to improved quality of life, reduced risk of cardiovascular diseases (CVD), and increased survival rates compared to ESRD patients treated using other methods.[1] One of the greatest challenges in transplant medicine has been to develop procedures aimed at reducing the risk of transplant rejection.[2] At present, acute rejection episodes following kidney transplantation are observed significantly less often. However, long-term observations suggest that during 10-year follow-up of kidney transplantation loss of function affects approximately 50% of transplanted organs.[3]

Administration of immunosuppressants following kidney transplantation is an obligatory element of therapy aimed at suppressing the body's immune response which may result in transplant rejection.[4,5] Immunosuppressants cause a range of adverse effects, hence it is necessary to keep the dosage at a level that will minimise the side effects but at the same time prevent the rejection of the transplanted organ by the immune system.[6] Despite the side effects, immunosuppressive treatment is also a necessity in female kidney transplants recipients who are pregnant.[5] But while it is necessary, immunosuppressive therapy during pregnancy raises concerns about the influence of the medication on fertility, pregnancy, and foetal development, as well as some long-term effects on offspring.[7]

Teratogenicity is listed as one of the side effects of immunosuppressants.[5] Immunosuppressive drugs and their metabolites cross the placental barrier and are transferred from the mother to the foetus.[5,8] Their numerous teratogenic effects include mutagenic effects (causing chromosomal aberrations), organ-specific

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toxicity, structural malformations in the foetus, and intrauterine growth retardation.[5,8] The adverse effects of immunosuppressive therapy increase the risk of premature birth or even spontaneous abortion.[5,8] A normal pregnancy, free from such risk factors as proteinuria, elevated creatinine levels ≥1.4 mg/dL or pharmacologically treated hypertension, does not increase the risk of transplant failure, compared to non-pregnant female transplant recipients.[9,10] Still, for many years transplantologists have been trying to establish the optimal time to conception after renal transplant. Currently in Europe, a two-year period between transplantation and conception is considered safe. According to the guidelines of the American Society of Transplantation, the best time to conceive is between one and two years post-transplantation.[5,10]

According to the drug classification of the US Food and Drug Administration (FDA), the majority of immunosuppressants fall into category C.[11] Nevertheless, as mentioned above, immunosuppressive treatment is continued during pregnancy in patients who received a kidney transplant. The currently adopted treatment regimen during pregnancy provides for the combination of: calcineurin inhibitors (tacrolimus or cyclosporine), azathioprine, and low dose prednisone. These drugs are regarded as relatively safe during gestation. On the other hand, immunosuppressants contraindicated during pregnancy, because of their teratogenic effects, include mycophenolate mofetil (MMF) and sirolimus (rapamycin).[5]

Fluoride (F) is a mineral which plays an active role in bone metabolism. It stimulates osteoblast proliferation, inhibits acid phosphatase and GTP-ase activity in osteoblasts, and also inhibits osteoclast proliferation, thus reducing bone demineralization. These processes induce bone tissue to grow and, therefore, F preparations have been used in some studies to manage glucocorticoid-induced osteoporosis.[12,13] Moreover, F exhibits anticariogenic effects through forming fluorohydroxyapatite and fluorapatite crystals, as well as being toxic to oral bacteria as an enzyme inhibitor.[14,15] On the other hand, F is known to be toxic to humans and high levels in the body may produce adverse effects in many organs.[15] In bones, overexposure to F causes crippling skeletal deformities by disrupting osteoclast and osteoblast metabolism,[16] while in teeth it leads to surface discoloration, damage and malformation of enamel.[16]

Although fluorine is neither an essential trace element for humans nor necessary for the development of healthy teeth and bones, topical fluoride on teeth is seen to have a protective effect against dental caries.[17] The beneficial effects of F on the human body can be observed only with low body concentrations of this mineral, and some immunosuppressants—such as prednisone, cyclosporine A, or tacrolimus—may disturb the mineral balance.[8,18] That is why in our present study, we have decided to investigate the influence of the three most common immunosuppressive therapies used after renal transplantation on bone and tooth F levels in mothers and their offspring. The study is also intended to tell us if the potential changes in elemental concentrations may affect human health.

MATERIAL AND METHODS

Characteristics of the study population: The study included 32 female Wistar rats (12 weeks old, mean body weight 230 g) and 8 Wistar males (Centre of Experimental Medicine, Medical University in Bialystok, Poland). The study was approved by the

Local Animal Research Ethics Committee in Szczecin (no 12/2013, dated 24 October 2013). All procedures were conducted in line with the current guidelines on the ethical and legal aspects of animal experimentation. The females participating in the study underwent a period of acclimatization, during which they were kept in single cells with a 12-hour light-dark cycle. The animals were fed Labofeed H (Morawski, Kcynia, Poland) and water *ad libitum*. Additionally, the animals received medication by oral gavage in specific combinations, with dosages based on literature data.[19-27] Daily doses were selected in such a way as to obtain plasma levels in the therapeutic range. All drugs were used in pharmaceutical form (Table).

Table. Dosage of the drugs used in the study.

The name of the active substance	Abbreviation	The name of the pharmaceutical form	Manufacturer	Dosage [mg/kg bw/d*]
Tacrolimus	Tc	Prograf	Astellas, USA	4
Mycophenolate mofetil	MMF	CellCept	Hoffmann- La Roche Ltd, Switzerland	20
Cyclosporine A	CsA	Sandimmun Neoral	Novartis, Switzerland	5
Everolimus	EVE	Certican	Novartis, Switzerland	0,5
Prednisone	Pr	Encorton	Polfa Warszawa, Poland	4

*bw/d = body weight per day

Female rats were divided into four groups:

- Control group (n = 8)—rats were given olive oil;
- Group B1 (n = 8)—rats were given CsA, MMF, and Pr;
- Group B2 (n = 8)—rats were given Tc, MMF, and Pr;
- Group B3 (n = 8)—rats were given CsA, EVE, and Pr.

Medication was administered to animals every 24 hr for 5 weeks: two weeks after acclimatisation up to the time of mating (when males and females 1:1 were placed in separate cages) and for three weeks of gestation. After mating, the females were transferred to separate cages. Once a week, the animals were weighed and dosage

was adapted to current weight. After delivery, during lactation, no medication was administered. 31 female rats completed the study. 69 pups were born in the control group, 13 pups in group B1 (group P), and one pup in group B3.

Due to the low number of pups, the experiment was repeated with dosage reduced by half. Only the steroid (prednisone) dosage was left at the same level. The study included eight female rats, 12 weeks of age. The rats were divided into three groups:

• Group Y (n = 2)—rats were given: CsA 2.5 mg/kg bw/d, MMF 10 mg/kg bw/d, and Pr 4 mg/kg bw/d;

• Group X (n = 3)—rats were given: Tc 2 mg/kg bw/d, MMF 10 mg/kg bw/d, and Pr 4 mg/kg bw/d;

• Group Z (n = 3)—rats were given: CsA 2.5 mg/kg bw/d, EVE 0.25 mg/kg bw/d, and Pr 4 mg/kg bw/d.

63 pups were born: 24 in group Y, 32 in group X, and 7 in group Z. The young rats were killed at 8 weeks after birth (12 rats in group Y, 12 in group X, and 7 in group Z). All rats were euthanized by intraperitoneal injection of sodium pentobarbital (Polpharma, Gdańsk, Poland) at 40 mg/kg body weight.

The study was approved by the Local Animal Research Ethics Committee in Szczecin (no 10/2014 and 11/2014, both dated 06 June 2014).

Preparation of samples for analysis and determination of fluoride content: Collected bone and tooth material was dried at 100ºC until dry mass was obtained. Afterwards, 10 mg samples were taken from the crushed material, 1 mL $HClO_4$ was added and then the sample was incubated for an hour at 95ºC. From the sample prepared in this way, 0.5 mL was mixed with 2 mL TISAB II and 2.5 mL 1M trisodium citrate solution. After mixing, F concentrations were determined by potentiometric analysis using an ion-selective electrode (Orion 9409 BN, Thermo Scientific, USA).[28]

Statistical analysis: Statistical analysis was performed using Statistica 13.0 (StatSoft, Poland). Data were tested for normal distribution using the Shapiro-Wilk test. Since data distribution deviated from a normal distribution, to examine the significance of differences between groups the non-parametric Mann-Whitney U test was employed.

RESULTS

The mean bone F concentration in the control group amounted to 91 mg/kg. In the analysis of mean F concentrations in the bones of mother rats exposed to immunosuppressive therapies, the highest mean F level was observed in group B1 exposed to CsA, MMF, and Pr (227 mg/kg), up by approximately 150% from the F level in the control group. In group B2 (Tc, MMF, and Pr), the mean F concentration amounted to 169 mg/kg, which is approximately 86% more than in the control group. The average F level in group B3 (CsA, EVE, and Pr) amounted to 191 mg/kg and was approximately 110% higher than the F level in the control group.

Statistical analysis confirmed the statistical significance of the differences in bone F content between the control group and each of the study groups—B1, B2, and B3 (Figure 1).

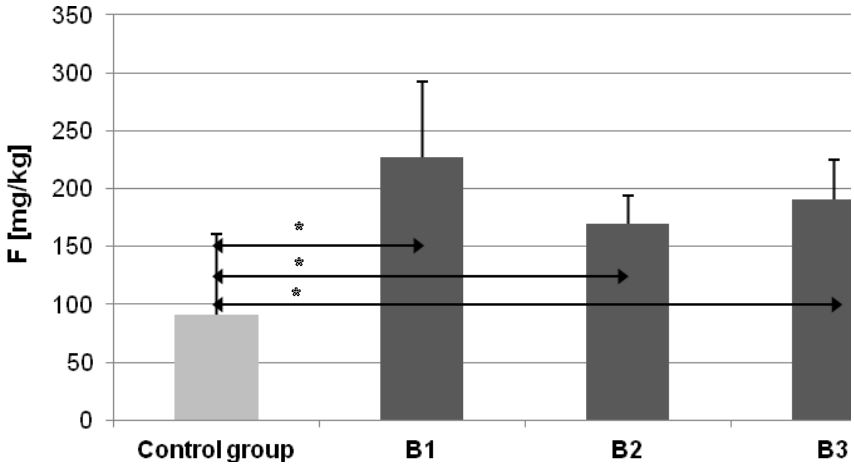

Figure 1. Mean fluoride concentration (and SD) in the bones of the female rats subjected to immunosuppressive therapies. B1—female rats receiving cyclosporine (CsA, 5 mg/kg bw/d), mycophenolate mofetil (MMF, 20 mg/kg bw/d), and prednisone (Pr, 4 mg/kg bw/d); B2—female rats receiving tacrolimus (Tc, 4 mg/kg bw/d), MMF (20 mg/kg bw/d), and Pr (4 mg/kg bw/d); B3—female rats receiving CsA (5 mg/kg bw/d), everlimus (EVE, 0,5 mg/kg bw/d), and Pr (4 mg/kg bw/d). Statistically significant differences: *p≤0.05.

In the next step of the study, we examined bone F levels in the offspring of the females subject to immunosuppression.

The next chart presents average F concentrations in the bones of the offspring of mothers exposed to immunosuppressive therapies (Figure 2).

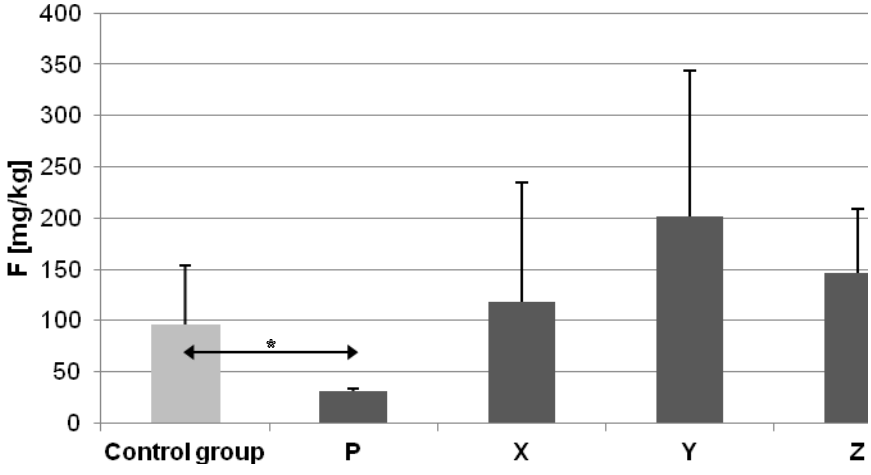

Figure 2. Mean fluoride concentrations (and SD) in the bones of offspring of mothers exposed to immunosuppressive therapies. P—offspring of mothers receiving cyclosporine (CsA, 5 mg/kg bw/d), mycophenolate mofetil (MMF, 20 mg/kg bw/d), and prednisone (Pr, 4 mg/kg bw/d); X—offspring of mothers receiving tacrolimus (Tc, 2 mg/kg bw/d), MMF (10 mg/kg bw/d), and Pr (4 mg/kg bw/d); Y–offspring of mothers receiving Pr (4 mg/kg bw/d), CsA (2,5 mg/kg bw/d), and MMF (10 mg/kg bw/d); Z–offspring of mothers receiving CsA (2,5 mg/kg bw/d), everlimus (EVE, 0,25 mg/kg bw/d), and Pr (4 mg/kg bw/d). Statistically significant differences: *p≤0.05.

The average F level in the offspring group P, whose mothers were exposed to CsA, MMF, and Pr (at full doses) amounted to 31 mg/kg and was statistically significantly lower (by approximately 68%) compared to the control group (97 mg/kg). In the offspring of mothers receiving half the dose of immunosuppressants the average F concentrations were higher than in the control group. The highest average F content, amounting to 202 mg/kg, was observed in the offspring in group Y, i.e., where the mothers were exposed to the combination of: CsA 2.5 mg/kg bw/d, MMF 10 mg/kg bw/d, and Pr 4 mg/kg bw/d. In group Z (mothers administered CsA 2.5 mg/kg bw/d, EVE 0.25 mg/kg bw/d, and Pr 4 mg/kg bw/d) the average bone level of F was 146 mg/kg and in group X (mothers administered Tc 2 mg/kg bw/d, MMF 10 mg/kg bw/d, and Pr 4 mg/kg bw/d) it was 118 mg/kg. However, due to large standard deviations observed in the control group and groups Y, X, and Z, statistical significance was not demonstrated for groups Y, X, and Z compared to the control group (Figure 2).

In the next step of the study, F concentrations were examined in the teeth of female rats subject to immunosuppressive therapies (Figure 3).

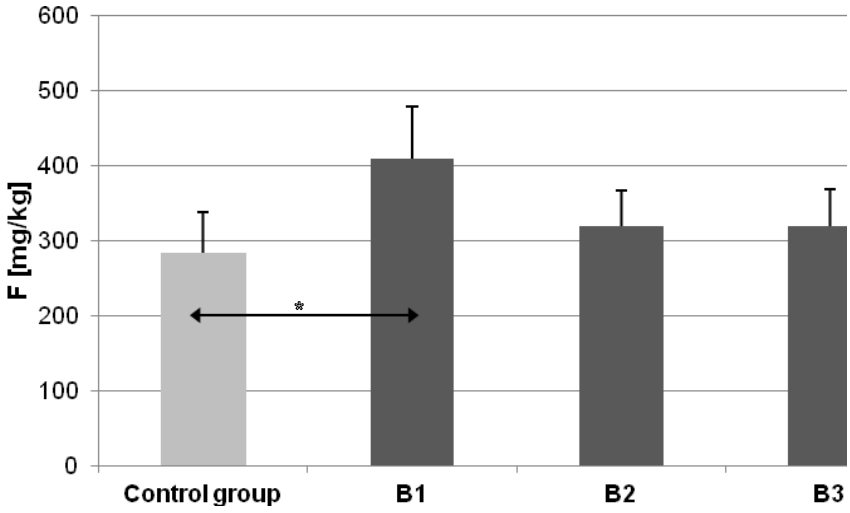

Figure 3. Mean fluoride concentrations (and SD) in the teeth of the female rats subjected to immunosuppressive therapies. B1—female rats receiving cyclosporine (CsA, 5 mg/kg bw/d), mycophenolate mofetil (MMF, 20 mg/kg bw/d), and prednisone (Pr, 4 mg/kg bw/d); B2—female rats receiving tacrolimus (Tc, 4 mg/kg bw/d), MMF (20 mg/kg bw/d), and Pr (4 mg/kg bw/d); B3—female rats receiving CsA (5 mg/kg bw/d), everlimus (EVE, 0,5 mg/kg bw/d), and Pr (4 mg/kg bw/d). Statistically significant differences: *$p \leq 0.05$.

The highest mean F content, amounting to 409 mg/kg, was observed in the group of mothers exposed to CsA, MMF, and Pr at full doses (Group B1). The F level in that group was higher than that found in the control group, amounting to 283 mg/kg, by approximately 45%. Statistical analysis demonstrated a statistically significant difference between group B1 and the control group ($p \leq 0.05$). F levels in the teeth of mothers from the control group and study groups B2 (319 mg/kg), and B3 (320 mg/kg) were similar.

Analysis of F levels in the tooth material collected from the offspring of mothers subjected to immunosuppressive therapies demonstrated that the highest tooth F content, amounting to 293 mg/kg, was noted in group X, that is the offspring of

mothers receiving Tc 2 mg/kg bw/d, MMF 10 mg/kg bw/d, and Pr 4 mg/kg bw/d. In comparison with the control group, however, no statistically significant differences were observed (Figure 4).

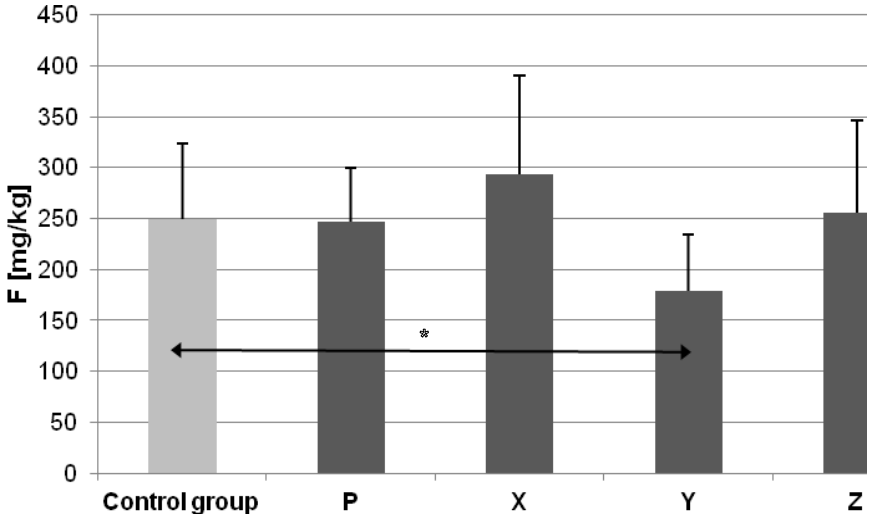

Figure 4. Mean fluoride concentrations (and SD) in the teeth of the offspring of mothers subjected to immunosuppressive therapies. P–offspring of mothers receiving cyclosporine (CsA, 5 mg/kg bw/d), mycophenolate mofetil (MMF, 20 mg/kg bw/d), and prednisone (Pr, 4 mg/kg bw/d); X–offspring of mothers receiving tacrolimus (Tc, 2 mg/kg bw/d), MMF (10 mg/kg bw/d), and Pr (4 mg/kg bw/d); Y–offspring of mothers receiving Pr (4 mg/kg bw/d), CsA (2,5 mg/kg bw/d), and MMF (10 mg/kg bw/d); Z–offspring of mothers receiving CsA (2,5 mg/kg bw/d), everlimus (EVE, 0,25 mg/kg bw/d), and Pr (4 mg/kg bw/d). Statistically significant differences: *$p \leq 0.05$.

In turn, the lowest F content, amounting to 178 mg/kg, was found in the teeth of the rats in group Y, that is the offspring of mothers administered CsA 2.5 mg/kg bw/d, MMF 10 mg/kg bw/d, and Pr 4 mg/kg bw/d. Statistical analysis confirmed that the differences in F concentrations between group Y and the control were statistically significant ($p \leq 0.05$).

Tooth F concentrations in the rats from groups P and Z, that is the offspring of mothers receiving respectively CsA 5 mg/kg bw/d, MMF 20 mg/kg bw/d, and Pr 4 mg/kg bw/d; and CsA 2.5 mg/kg bw/d, EVE 0.25 mg/kg bw/d, and Pr 4 mg/kg bw/d reached 246 mg/kg (group P) and 256 mg/kg (group Z) and were therefore similar to the F levels observed in the teeth of the control group (249 mg/kg).

DISCUSSION

Bone loss in the early post-transplant period has been largely attributed to the effects of high-dose glucocorticoid treatment.[29,30] As a result, even 8 years after kidney transplantation, about 50% of the recipients continue to have low BMD (bone mass density), contributing to an increased risk of fractures.[29] Strong demineralization and bone loss caused by immunosuppressive medication is often the price that has to be paid for preventing transplant failure.[29] The introduction of nonsteroidal immunosuppressive drugs has helped create more personalised immunosuppressive regimens which offer a possibility of limiting the adverse effects on glucocorticoids on hard tissues. Still, data regarding the different effects of

nonsteroidal immunosuppressive drugs on human bone metabolism are limited.[31] It also needs to be noted that therapies are most commonly based on a combination of several drugs which may interact with each other.[5] The data published to date fail to provide information regarding changes in the concentrations of macro- and microelements (F being one of them) in the bone tissue of pregnant women treated according to various therapeutic models in long-term immunosuppression.[5] Hence, in our attempt to explore the influence of immunosuppression on F levels in hard tissues of female rats and their offspring, in each of the study groups a combination of three drugs was employed: two that are regarded as safe to use during pregnancy and one that is contraindicated.[5]

In one prospective randomized study, kidney recipients treated with CsA and glucocorticoids were found to have significantly decreased BMD in the first 18 months of therapy. The exclusion of glucocorticoids led to a marked improvement in BMD, which suggests that CsA has no negative effect on bone mineralization.[32] The study by Cvetkovic et al. shows that both CsA and Tc cause osteopenia in rats,[33] while Inoue et al. confirm CsA-induced osteopenia in rats, but with no significant bone loss following the administration of Tc. They also found that Tc leads to elevated IGF-I levels, which may have a positive effect on bone tissue.[34] Also in the paper by Goffin et al. it was noted that the application of Pr and CsA in patients following kidney transplantation has a significant effect on bone loss, while therapy based on Pr and Tc leads to higher bone mass and increased bone turnover.[35] In a study conducted among human kidney transplant recipients it was observed that sirolimus, too, may have a positive effect on bone mineral density. The drug is associated with lower readings of factors reflecting osteoclast activity in serum in patients, and it also slows down maturation of osteoclasts. EVE directly inhibits bone resorption by suppressing osteoclast precursors, which may contribute to bone accretion, but the drug was also observed in the study to inhibit osteoblast differentiation.[36] In a rat study conducted by Dissanayake et al., MMF, like EVE, did not cause bone loss, but it affected osteocalcin levels, which proves its modulatory effect on bone metabolism.[37] In our study, mother rats receiving various combinations of immunosuppressive drugs had significantly higher bone F levels compared to the control group. F in low concentrations is known to contribute to bone accretion by stimulating osteoblasts and inhibiting osteoclast activity, but higher F levels may lead to pathologies in bone tissue. As a result of F exposure, bone crystallises in peripheral areas, while its central part remains amorphous, leading to the formation of deformed fluorohydroxyapatite and fluorapatite crystals. In addition, F-induced osteogenesis does not lead to the formation of new trabeculae, reducing bone strength and increasing the risk of fractures.[38] Studies by other authors demonstrated moreover that the increase in bone mineral density following the use of NaF for preventing bone loss in immunosuppressive treatment did not lower the risk of fractures[39,40] and in some cases even increased it.[12]

In young rats whose mothers were exposed to full-dose immunosuppressive medication, a significant drop in bone F content was observed compared to the control, while in the offspring whose mothers were exposed to half doses, bone F concentrations were higher. This was confirmed in the study by del Pozo et al.[41] which investigated the influence of CsA on bone mineral density in young rats. The authors demonstrated that doses of 5 and 15 mg/kg do not affect bone mineral

density, while 30 mg/kg leads to demineralization.[41] Bones of young rats are particularly sensitive to immunosuppressive medication because of the high metabolic activity in bone tissue. Katz et al. in their study on the influence of CsA on the bones of young rats observed increased bone turnover, which was accompanied by elevated BGP levels in serum and bone loss.[42] The decreased F levels in the bones of the offspring of mothers exposed to full dose medication observed in our study was likely to be caused by increased bone resorption resulting from excessive dosage.

By influencing bone metabolism, immunosuppressive drugs may also affect periodontal bones. In the study by dos Santos et al. it was noted that glucocorticoids, CsA, Tc, and sirolimus may cause periodontal bone resorption and, consequently, lead to tooth mobility.[43] No effects were observed following the use of MMF.[43] Additionally, some immunosuppressants may act directly on dental tissue. Studies on children following liver transplantation show that CsA and Tc may cause enamel hypoplasia and discoloration, as well as increase severity of caries.[44] In our study, the administration of full-dose MMF, CsA, and Pr led to a significant increase in F levels in rat teeth compared to the control group. The above mentioned changes of F metabolism in humans receiving immunosuppressive treatment may be associated with an increased risk of dental fluorosis, if given while the teeth are still forming up to the age of approximately 8 yr.[14] In the group of rat offspring exposed to Pr and reduced doses of CsA and MMF, significantly lower tooth F levels were observed. It should be noted however that in the offspring exposed to full doses of Pr, CsA, and MMF accumulation of F in teeth was comparable to that in the control group. Loss of bone mineral mass is known to be caused mainly by glucocorticoids.[29] However, in a randomised, multi-centre study it was demonstrated that a reduced dose of glucocorticoids combined with full doses of CsA and MMF led to an improvement in mineral density compared to the group receiving a standard dose of glucocorticoids.[45] Potentially, higher doses of MMF and CsA protect against mineral loss, including F.

CONCLUSIONS

In conclusion, immunosuppressive therapies used in kidney transplant recipients affect F metabolism in bones both in mothers and their offspring. Elevated bone F levels in transplant recipients may impair bone strength, while children of people receiving therapies may be exposed to major mineral loss. A therapy based on prednisone, MMF, and CsA also led to a significant upswing in tooth F levels, which may increase the risk of developing dental fluorosis.

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