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ORIGINAL PAPER

IMMUNOSUPPRESSIVE TREATMENT DURING PREGNANCY AS A POTENTIAL FACTOR CHANGING MAGNESIUM, CALCIUM AND PHOSPHORUS LEVELS IN HARD TISSUES OF FEMALE RATS AND THEIR OFFSPRING*

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Abstract

Immunosuppressive therapy is necessary to prevent transplant rejection, also in the case of pregnant transplant recipients, which means that the medications may influence foetal development. An ideal immunosuppressive regimen should provide for excellent immunosuppression with minimal or no side effects. Yet, current immunosuppressive therapy regimens commonly used in clinical applications fail to meet this criterion. One of the complications caused by immunosuppressive drugs are mineralisation disorders in hard tissues. Therefore, in this study, we evaluated the impact of three regimens of immunosuppressive therapy used after renal transplantation, containing medications which are indicated (prednisone, cyclosporine A (CsA), tacrolimus (Tc)) and contraindicated (mycophenolate mofetil (MMF), everolimus) during pregnancy on the concentrations of essential minerals, calcium (Ca), phosphorus (P) and magnesium (Mg), affecting normal bone formation. The samples were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES, ICAP 7400 Duo, Thermo Scientific) equipped with a concentric nebuliser and a cyclonic spray chamber. The immunosuppressive regimens under study had no effect on the levels of Mg and P, but they did contribute to increased bone Ca levels in the mothers in the group receiving Tc, MMF and prednisone and group receiving CsA, everolimus and prednisone. In the offspring of tested mother rats, immunosuppressive therapies may affect Mg levels in hard tissues. The immunosuppressive regimens administered

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at therapeutic doses are harmful to rat foetuses as evidenced by the small number or lack of offspring in the tested groups.

Keywords: immunosuppressive therapy, pregnancy, elements content, bone, tooth.

INTRODUCTION

Organ transplantation is a generally accepted treatment method for organ failure (Kalantar-Zadeh et al. 2012). When successful, it can reverse many complications caused by organ dysfunction, but bone metabolism and mineralisation disorders continue to be problematic. These disorders may be caused by the underlying disease (in the case of end-stage renal disease) and by the use of immunosuppressant medications to prevent transplant rejection (Coco 2003, Kalantar-Zadeh et al. 2012). Disorders in bone remodelling are mainly related to bone formation and mineralisation, with persistently elevated bone resorption. It leads to alterations in bone metabolism markers (PTH, alkaline phosphatase, vitamin D and FGF-23) and contributes to the loss of minerals (Ca, P, Mg) from hard tissues, increasing the rate of bone loss and risk of fracture (Bellorin-Font et al. 2003, Kalantar-Zadeh et al. 2012). The decrease in bone mineral density (BMD) is most pronounced in the initial 6-18 months after renal transplantation, and is attributed mainly to the osteolytic effects of high doses of glucocorticoids administered in the early post-transplantation period (Grotz et al. 1995). In the long-term follow-up (more than 12 months after renal transplantation), researchers observed less pronounced bone loss (Yazawa et al. 1998) and even bone mass increase (Vicente-Rodríguez et al. 2008). Nevertheless, at about 8 years post-surgery more than 50% of kidney transplant recipients present with osteoporosis, which shows that the adverse changes in bone metabolism persist long after organ transplantation (Westenfeld et al. 2011).

Immunosuppressive therapy is necessary to prevent transplant rejection, also in the case of pregnant transplant recipients, which means that the medications may influence foetal development (Kabat-Koperska et al. 2016). Immunosuppressive medications and their active metabolites cross the placental barrier and may exert teratogenic and mutagenic effects on the foetus as well as cause organ-specific toxicity, leading to the emergence of structural and developmental defects. This results in an increased risk of premature birth and spontaneous abortion (Kabat-Koperska et al. 2016*a*,*b*). At present, it is believed that 2 years suffice for a safe interval between organ transplantation and conception, as by that time the pre-emptive antiviral therapy has been completed and the levels of immunosuppressive medication are low. However, recent studies have shown an increased risk of transplant failure in pregnant women in the first and second year post-transplantation, while pregnancy in the third post-transplant year was not associated with an increased risk of graft loss (Shah, Verma 2016). The regimen used in the immu-

nosuppressive therapy for pregnant transplant recipients is based on a combination of a calcineurin inhibitor (tacrolimus or cyclosporine), azathioprine and prednisone. In turn, because of the strong teratogenic effects, mycophenolate mofetil (MMF) and sirolimus (rapamycin) should be avoided (Kabat-Koperska et al. 2016b).

Ca, P and Mg are some of the essential minerals affecting normal bone formation (Vicente-Rodríguez et al. 2008). 99% of Ca, 85% of P and approx. 60% of Mg in the human body are found in hard tissues (Palacios 2006, Kovacs 2015). While kidneys and intestines control mineral metabolism in adults, they play a minor role in the foetus. Minerals are supplied to the foetus mainly via the placenta, which through active transport supplies minerals from the mother's circulation and carries away excess minerals and unnecessary products back to the mother. Adequate levels of minerals may be carried across the placenta even if the concentration of a given mineral in the mother's body is low, which may potentially be an additional factor adversely affecting the mother's mineral metabolism (Coco 2003, Kalantar-Zadeh et al. 2012, Kovacs 2015). On the other hand, foetal serum levels of Ca, P and Mg are much higher than those in the mother. Ca levels are up by 0.30-0.50 mmol dm^{-3}, P by approx. 0.5 mmol dm^{-3}, and Mg by 0.05 mmol dm^{-3}, probably because of the large demand for these minerals in the rapidly growing foetal skeletal system over the relatively short period of intrauterine development (Kovacs 2015). Mineral transport across the placenta, mineral regulation in the foetal serum as well as foetal bone development and mineralisation are mainly dependent upon PTHrP (parathyroid hormone-related protein) and PTH (parathyroid hormone) (Kovacs 2015). Through its local effect on bone, PTHrP plays a key role as a regulator of chondrocyte life span in the developing skeletal system, while PTH – as a systemic hormone – is essential in hard tissue mineralisation by maintaining high Ca levels in the serum (Kovacs 2015, et al. 13). Under normal conditions, PTH levels in foetal blood remain low, while PTHrP levels may be 15 times higher (Kovacs 2015). Glucocorticoids bring down PTHrP levels (Kovacs 2015, Ahlström et al. 2009), which may induce premature chondrocyte apoptosis, accelerated mineralisation, abnormal differentiation of the cartilaginous skeleton, thereby leading to reduced limb length (Kovacs 2015). On the other hand, glucocorticoids may increase PTH levels, though the exact mechanism of their influence is unclear (Rubin, Bilezikian 2002, Patschan et al. 2001, Stempfle et al. 2002). It was demonstrated that calcineurin inhibitors (calcineurin; protein phosphatase 2B (PP2B)) may increase PTH levels in transplant recipients (Blaslov et al. 2014). It is known that bone metabolism is different in rats and humans, therefore the effects of immuno-suppressive drugs may be different. On the other hand, due to the toxic effects of immunosuppressive drugs on foetus such as MMF or mTOR inhibitors, these drugs can be examined only in an animal model, therefore in the present study, we evaluated the impact of three immunosuppressive regimens used after kidney transplantation, containing medications which

are indicated (prednisone, cyclosporine A (CsA), tacrolimus (Tc)) and contra-indicated (mycophenolate mofetil (MMF), everolimus) during pregnancy (Kabat-Koperska et al. 2016b) on the concentrations of Ca, P and Mg in the hard tissues of mother rats and their offspring.

MATERIAL AND METHODS

Characteristics of the study population

The study was conducted on 32 female Wistar rats at twelve weeks of age, with average body weight of 230 g, and 8 males for mating. The animals were acquired from a licensed breeder (Centre of Experimental Medicine, Medical University in Białystok, Poland). The study was approved by the Local Animal Research Ethics Committee in Szczecin (no 12/2013, dated 24 October 2013). The females underwent a period of acclimatisation, 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 (Katz et al. 1991, Viklický et al. 2001, Jolicoeur et al. 2003, Ma et al. 2006, van Westrhenen et al. 2007, Martinez-Palli et al. 2011, Kurdián et al. 2012, Piao et al. 2012, Sagiroglu et al. 2014). Eight rats comprising the control group were given carrier and olive oil. The medications were used in pharmaceutical form and the dosages were selected in such a way as to obtain plasma levels in the therapeutic range (Table 1).

Females participating in the study were divided into four groups – the control and three study groups receiving drug combinations. Each group consisted of eight female rats (n=8).

– group B1 – receiving CsA, MMF and prednisone;
– group B2 – receiving Tc, MMF and prednisone;
– group B3 – receiving CsA, everolimus and prednisone.

Table 1

The list of the medications used with dosages

Name of active substance	Summary	Name of the pharmaceutical form	Manufacturer	Dosage (mg kg^{-1} b.w./d.)
Takrolimus	Tc	Prograf	Astellas, USA	4
Mycophenolate mofetil	MMF	CellCept	Hoffmann – La Roche Ltd, Switzerland	20
Cyclosporine	Cs	Sandimmun Neoral	Novartis, Switzerland	5
Ewerolimus	EWE	Certican	Novartis, Switzerland	0.5
Prednizon	Pred	Encorton	Polfa Warszawa, Poland	4

Medications were administered to animals every 24 hours over a period of 5 weeks: two weeks after acclimatisation prior to mating – after placing males and females 1:1 in separate cages – and after mating for three weeks of gestation. After mating, the females were transferred to separate cages. Once a week, the animals were weighed and drug dosage was adapted to current weight. For the evaluation of drug concentrations in rats blood, we used a separate group of pregnant female rats (n=14) at the corresponding age. The concentration of CsA was determined with the Abbott AxSYM assay, which is based on fluorescence. To determine Tc level, we used the IMx assay based on a microparticle enzyme immunoassay. The concentration of everolimus was determined at the Laboratory of Mass Spectometry. After delivery, during lactation, no medication was administered, seen as women are advised not to breastfeed while taking immunosuppressants. 31 female rats completed the study, with 69 pups born in the control group, 13 pups in group B1 (group B21) and one pup in group B3.

Due to the small number of pups born in the study groups, the experiment was repeated with medication dosage reduced by half. Only the dosage of prednisone was left unchanged. The medication regimen remained the same as in the first part of the study. The study was approved by the Local Animal Research Ethics Committee in Szczecin (no 10/2014 and 11/2014, dated 06 June 2014). The study included eight female rats, at 12 weeks of age, assigned randomly to the following groups:

- group B11 (n=2) – receiving CsA (2.5 mg kg^{-1} per day), MMF (10 mg kg^{-1} per day) and prednisone (4 mg kg^{-1} per day);
- group B12 (n=3) – receiving Tc (2 mg kg^{-1} per day), MMF (10 mg kg^{-1} per day) and prednisone (4 mg kg^{-1} per day);
- group B13 (n=3) – receiving CsA (2.5 mg kg^{-1} per day), everolimus (0.25 mg kg^{-1} per day) and prednisone (4 mg kg^{-1} per day).

A total of 63 pups were born: 24 in group B11, 32 in group B12, and 7 in group B13. Juvenile rats were killed at 8 weeks after birth (12 rats in group B11, 12 in group B12, and 7 in group B13). All rats were euthanised by intraperitoneal injection of sodium pentobarbital (Polpharma, Gdańsk, Poland) at 40 mg kg^{-1} body weight.

Sample preparation and decomposition

Rat bone and tooth material collected during necropsy was dried at 100°C until dry mass was obtained. Dry tissue was crushed with an agate mortar and pestle and 100 mg samples were weighed into plastic vials and labelled. After preparation, the samples were subjected to a microwave decomposition procedure using a microwave digestion system (MARS 5, CEM). To this end, 2 ml of 65% HNO_3 (Suprapur, Merck) was poured on top of the 100 mg samples, transferred into Teflon vessels and placed in the microwave digester. The digestion process was divided into two stages – the first (15 min), during which the samples were gradually heated up to

180°C, and the second, lasting 20 min, during which the temp. was maintained at 180°C.

Elemental composition analysis

After digestion, samples were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES, ICAP 7400 Duo, Thermo Scientific) equipped with a concentric nebuliser and a cyclonic spray chamber. Each sample was diluted 20-fold, and then an internal standard was added to 500 μL of the obtained solution: yttrium at the final concentration of 0.5 mg L^{-1}. 1 ml of 1% Triton (Triton X-100, Sigma) was also added as a sample emulsifier to facilitate calibration against inorganic aqueous standard solutions. The samples were diluted with 0.075% HNO_3 (Suprapur, Merck) up to the final volume of 10 mL, and stored in the fridge (4-8°C) until analysis. The blank test was prepared according to the same procedure, with the study sample replaced by 250 μL of nitric acid (V). The calibration curve was prepared using multi-element standard solutions (ICP multi-element standard solution IV, IX and XVI, Merck). All solutions were made using deionised water (Direct Q UV, Millipore, approx.18.0 MΩ).

Statistical analysis

Statistical analysis was carried out using the Statistica software package (StatSoft, Poland), and the graphic presentation of results was prepared in Excel 2010. Since the data did not follow a normal distribution, the non-parametric Kruskal-Wallis test was used in further statistical analysis. Results of the calculations were verified with p-value ≤ 0.05 adopted as the statistical significance threshold.

RESULTS

Effects of immunosuppressive therapy on the levels of Ca, Mg and P in the bones of mother rats

The mean Ca level in the bones of mother rats exposed to immunosuppressive therapy in the control group amounted to 257 mg g^{-1} (SD=17.4433). The highest mean concentration of Ca was observed in group B3, receiving CsA, everolimus and prednisone – 286 mg g^{-1} (SD=23.6556). This result is significantly higher compared to the control group (p=0.018). In group B2, receiving Tc, MMF and prednisone, the mean Ca level amounted to 278 mg g^{-1} (SD=21.2939), and in this case, too, the difference compared to the control group was statistically significant (p=0.04). The mean Ca level in group B1, exposed to CsA, MMF and prednisone, was 268 mg g^{-1} (SD=33.5110). The differences between group B1 and control were not statistically significant (p>0.05) – Figure 1*a*.

The highest mean concentration of Mg was observed in the control group (5.1 mg g^{-1}, SD=0.7818) and group B1 (5 mg g^{-1}, SD=0.7643). In group B2, the mean Mg level amounted to 4.6 mg g^{-1} (SD=0.5119), and in group B3 – 4.7 mg g^{-1} (SD=0.4712) – Figure 1*b*. Statistical analysis failed to identify significant differences between the groups in this case (p>0.05) (Figure 1*b*).

The samples were also analysed for the content of P, which was similar across all the groups (control: 139 mg g^{-1}, SD=14.7484, group B1: 139 mg g^{-1}, SD=17.2622, group B2: 138 mg g^{-1}, SD=8.2038, group B3: 140 mg g^{-1}, SD=7.7598). In the case of this mineral, no statistically significant differences were noted between the analysed groups (p>0.05) – Figure 1*c*.

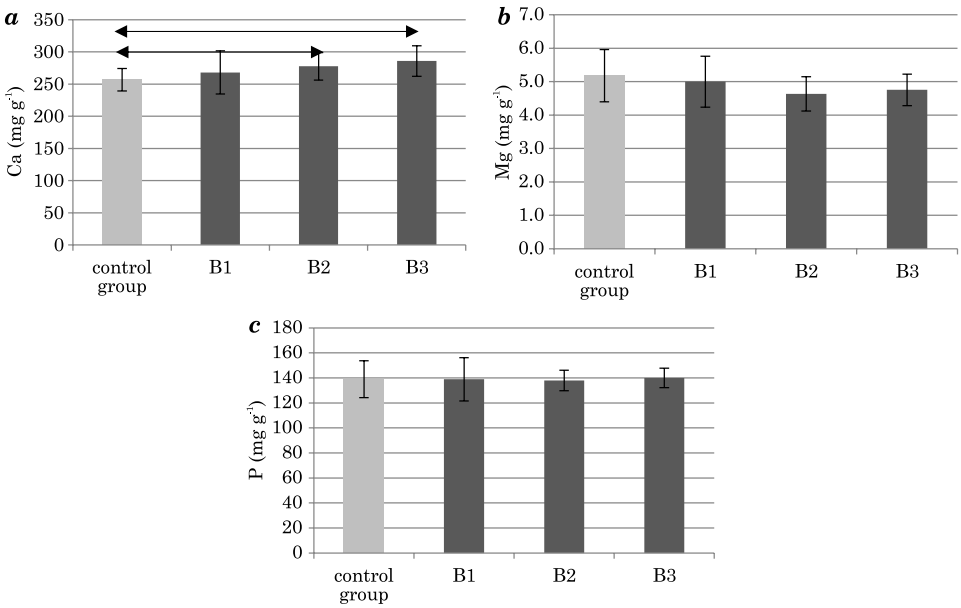

Fig. 1. Mean concentration and SD of calcium (*a*), magnesium (*b*) and phosphorus (*c*) in bones of rat mothers after immunosuppressive therapy: B1 – rat mothers receiving CsA (5 mg kg^{-1} per day), MMF (20 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day), B2 – rat mothers receiving Tc (4 mg kg^{-1} per day), MMF (20 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day), B3 – rat mothers receiving CsA (5 mg kg^{-1} per day), Everolimus (0.5 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day). Statistically significant differences – $p \leq 0.05$

Part two of the study focused on the analysis of Ca, P and Mg levels in the bones of the offspring of the females receiving immunosuppressive therapy during pregnancy.

The mean Ca level in the group exposed to the full doses of CsA, MMF and prednisone (group B21) was 242 mg g^{-1} (SD=10.9646), while in the offspring of mothers receiving half the dosage of the same immunosuppressive medications (group B11), the mean Ca level amounted to 256 mg g^{-1} (SD=20.4903). The highest mean concentration of Ca, amounting to 261 mg g^{-1} (SD=39.3236), was observed in the offspring from group B13, exposed via the mothers to CsA, everolimus and prednisone. In group B12 (mothers given Tc,

MMF and prednisone), the mean bone Ca level amounted to 250 mg g^{-1} (SD=12.1697). In the control group, the mean Ca level amounted to 245 mg g^{-1} (SD=16.0464). Statistical analysis failed to identify statistically significant differences between the control group and study groups (p>0.05) – Figure 2*a*.

The highest mean bone Mg level in the offspring of females subjected to immunosuppressive therapy was found in group B21 and amounted to 5.8 mg g^{-1} (SD=0.5602). The result was significantly higher compared to the control (p=0.04), wherein it amounted to 5 mg g^{-1} (SD=0.7399). The mean Mg level in group B11 was 5.6 mg g^{-1} of bone (SD=0.6023). This figure was also significantly higher compared to the control. The mean Mg level in group B12 amounted to 5.2 mg g^{-1} (SD=0.6441) and in group B13 – 5.4 mg g^{-1} (SD=0.6524). In the comparison of these two groups to the control, no statistical significance was identified (p>0.05) – Figure 2*b*.

The mean bone content of P in the offspring of mother rats exposed to immunosuppressive therapies in the control group amounted to 132 mg g^{-1} (SD=12.3213). The highest mean concentration of P was observed in group B21 and amounted to 141 mg g^{-1} (SD=10.0047), in group B11 – 139 mg g^{-1} (SD=12.0270), in group B12 – 130 mg g^{-1} (SD=12.3558), and in group B13 –

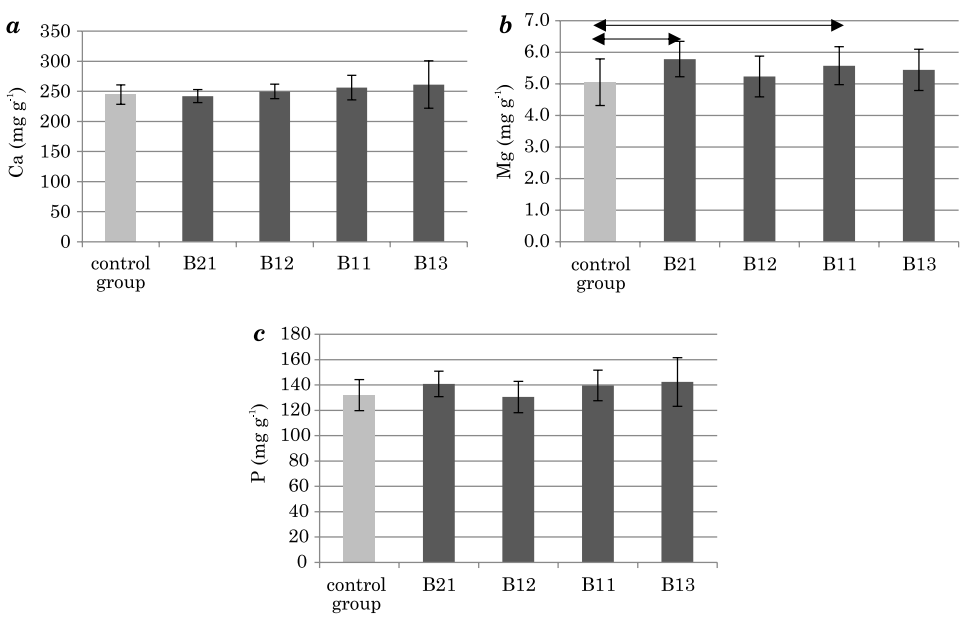

Fig. 2. Mean concentration and SD of calcium (*a*), magnesium (*b*) and phosphorus (*c*) in bones of offspring of rat mothers receiving immunosuppressive therapy: P – offspring of rat mothers receiving CsA (5 mg kg^{-1} per day), MMF (20 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day), X – offspring of rat mothers receiving Tc (2 mg kg^{-1} per day), MMF (10 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day), Y – rat mothers receiving CsA (2.5 mg kg^{-1} per day), MMF (10 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day); Z – rat mothers receiving CsA (2.5 mg kg^{-1} per day), Everolimus (0.25 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day). Statistically significant differences – $p \leq 0.05$

142 mg g^{-1} (SD=19.1621). For this mineral, no statistically significant differences were identified, either (p>0.05) – Figure 2*c*.

Effects of immunosuppressive therapy on the levels of Ca, Mg and P in teeth

The analysis of teeth of the mothers receiving immunosuppressive therapies demonstrated that the highest mean Ca level was observed in the control group, amounting to 316 mg g^{-1} (SD=38.3803), whereas the lowest concentration, at 293 mg g^{-1} (SD=31.7500), was found in group B3, receiving CsA, everolimus and prednisone. The Ca level in group B1, receiving CsA, MMF and prednisone, was 296 mg g^{-1} of tissue (SD=37.5828), and in group B2, receiving Tc, MMF and prednisone, it reached 304 mg g^{-1} (SD=8.5392). Ca levels were comparable across all the studied groups, hence statistical analysis failed to identify significant differences (p>0.05) – Figure 3*a*.

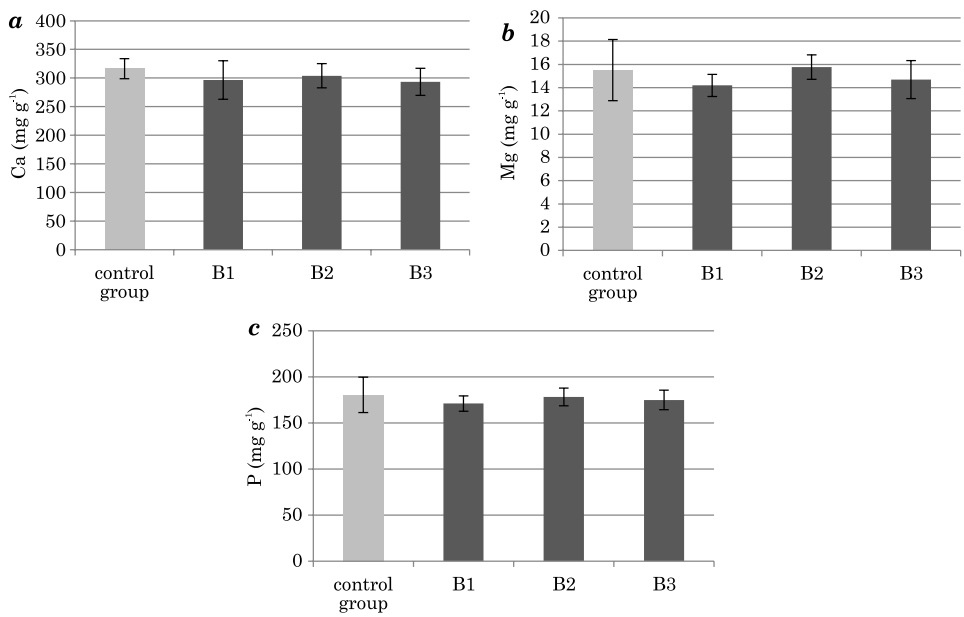

Fig. 3. Mean concentration and SD of calcium (*a*), magnesium **(***b*) and phosphorus (*c*) in teeth of rat mothers after immunosuppressive therapy: B1 – rat mothers receiving CsA (5 mg kg^{-1} per day), MMF (20 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day), B2 – rat mothers receiving Tc (4 mg kg^{-1} per day), MMF (20 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day), B3 – rat mothers receiving CsA (5 mg kg^{-1} per day), Everolimus (0.5 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day). Statistically significant differences – $p \leq 0.05$

The mean Mg level among mothers receiving immunosuppressive therapies in the control group amounted to 15.5 mg g^{-1} (SD=2.6310). A slightly higher concentration was observed in group B2, amounting to 15.8 mg g^{-1} (SD=1.0508), whereas lower levels than in the control group were determined in groups B1 (14.2 mg g^{-1}, SD=0.9553) and B3 (14.7 mg g^{-1}, SD=1.6305).

Statistical analysis failed to identify any significant differences (p>0.05) – Figure 3*b*.

Similar to Ca, the P content among mothers receiving immunosuppressive therapies was highest in the control group and amounted to 180 mg g^{-1} (SD=19.2743). Marginally lower levels were observed in groups B2 (178 mg g^{-1}, SD=9.5681), B3 (175 mg g^{-1}, SD=10.6082), and B1 (171 mg g^{-1}, SD=8.3259). In this case, too, statistical analysis failed to identify significant differences between the control and study groups (p>0.05) – Figure 3*c*.

The analysis of tooth tissue for Ca levels in the offspring of mothers receiving immunosuppressive therapies demonstrated that the highest mean Ca level, amounting to 282 mg g^{-1} (SD = 36.2626), was found in the teeth of offspring in group B12. On the other hand, the lowest level of that mineral, at 242 mg g^{-1} (SD=20.2514), was observed in the teeth of offspring of the mothers given reduced doses of CsA, MMF and prednisone (Group B11). The tooth Ca level in the offspring from group B21 amounted to 278 mg g^{-1} (SD=43.6232), and in group B13 – 256 mg g^{-1} (SD=44.0925). Statistical analysis did not identify significant differences between the control (C = 271 mg g^{-1}, SD=38.1894) and study groups (p>0.05) – Figure 4*a*.

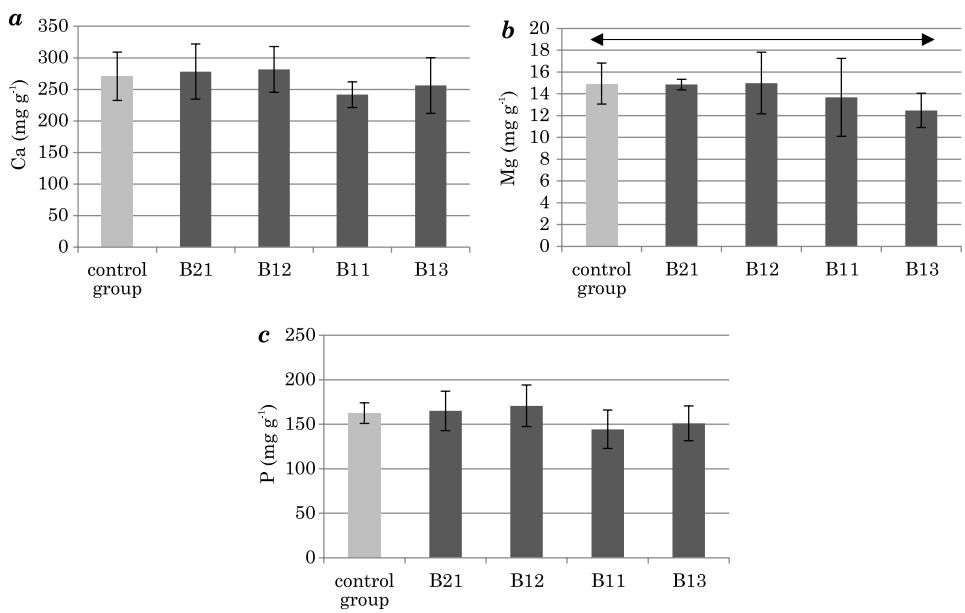

Fig. 4. Mean concentration and SD of calcium (*a*), magnesium (*b*) and phosphorus (*c*) in teeth of offspring of rat mothers receiving immunosuppressive therapy: P – offspring of rat mothers receiving CsA (5 mg kg^{-1} per day), MMF (20 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day), X – offspring of rat mothers receiving Tc (2 mg kg^{-1} per day), MMF (10 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day), Y – rat mothers receiving CsA (2.5 mg kg^{-1} per day), MMF (10 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day); Z - rat mothers receiving CsA (2.5 mg kg^{-1} per day), Everolimus (0.25 mg kg^{-1} per day), prednisone (4 mg kg^{-1} per day). Statistically significant differences – $p \leq 0.05$

The highest tooth Mg level was determined in the offspring from group B12, where it amounted to 15 mg g^{-1} (SD=2.8263). Marginally lower levels were observed in the control group (14.9 mg g^{-1}, SD=1.8895) and group B21 (14.8 mg g^{-1}, SD=0.4862). In turn, markedly lower Mg levels were observed in group B11 (13.7 mg g^{-1}, SD=3.5647) and in group B13 (12.4 mg g^{-1}, SD=1.5708). Due to the significant standard deviations, the difference in Mg levels between the control group and group B11 was not statistically significant (p>0.05), nevertheless a significant difference was identified in Mg content between the control and group B13 (p=0.01) – Figure 4*b*.

The mean P level in the control group was 162 mg g^{-1} (SD=11.6248). Higher P levels were obtained in group B12 (171 mg g^{-1}, SD=23.3493) and group B21 (165 mg g^{-1}, SD=22.1710), while lower P levels were found in group B13 (151 mg g^{-1}, SD=19.6578) and group B11 (165 mg g^{-1}, SD=22.1710), but the differences were not statistically significant (p>0.05) – Figure 4*c*.

DISCUSSION

An ideal immunosuppressive regimen should provide for excellent immunosuppression with minimal or no side effects. Yet, current immunosuppressive therapy regimens commonly used in clinical applications fail to meet this criterion. A promising regimen is the use of combination immunosuppressive therapy with low dosages, which would help minimise side effects while maintaining therapeutic efficacy (Goodman et al. 2001). Many studies demonstrated that responsibility for bone mineral loss lies mainly with glucocorticoids (Westenfeld et al. 2011, Weinstein 2011, Zhang 2012). One way to deal with this would be to include non-steroid immunosuppressive medications, which would help reduce the steroid-related side effects (Westenfeld et al. 2011). Regrettably, the mechanisms of action and effects of those drugs have not been fully explained to date (Kabat-Koperska et al. 2016*b*), and attempts to investigate their impact on the mineral density in hard tissues have yielded conflicting findings (Aroldi et al. 1991, Inoue et al. 2000). Moreover, the effect of a specific drug may differ in monotherapy and in combination therapy with other immunosuppressive medications (Kabat-Koperska et al. 2016*b*). The literature for the most part focuses on investigating the influence of one or two drugs, and there are scarce reports of changes observed with the implementation of therapy combining several drugs (Dounousi et al. 2015, Kabat-Koperska et al. 2016*b*). In an effort to find out if the studied regimens may affect the mineral content of hard tissues in the mothers and their offspring, and whether medications contraindicated in pregnancy in combination with other immunosuppressive medications may cause adverse effects for the foetus, each group was exposed to two drugs considered safe during pregnancy and one which

is contraindicated (in groups B1 and B2 – MMF, in group B3 – everolimus) – Kabat-Koperska et al. (2016*b*).

Immunosuppressive drugs, such as glucocorticoids, cyclosporine A (CsA), azathioprine, tacrolimus (FK 506), and recently put into use mycophenolate mofetil and sirolimus (SRL; rapamycin) are used to prevent transplant rejection (Goodman et al. 2001). Bone resorption is a common phenomenon following organ transplantation, and its mechanism is complex. Apart from the direct effect of immunosuppressants on bone cells, concomitant hyperparathyroidism and elevated PTH levels, reduced amounts of skeletal growth factors, inhibited secretions of adrenocorticotropic hormone and gonadotropin, which consequently suppress the release of gonadal and adrenal hormones, impairment of kidney function brought about by drug nephrotoxicity, all indirectly contribute to bone loss (Stempfle et al. 2002). In the study by Stempfle et al., it was demonstrated that glucocorticoids, cyclosporine A and tacrolimus have an adverse effect on bone mineral density. What is more, tacrolimus, by exerting direct toxic effect on bone cells and secreting local autocrine factors, as well as acting indirectly through cytokine modulation, caused a much more rapid bone loss than glucocorticoids did (Stempfle et al. 2002). No negative effects were observed after administering azathioprine or MMF (Stempfle et al. 2002). The negative effects of glucocorticoids, tacrolimus and CsA on bone tissue, and the lack of side effects with the use of azathioprine and MMF were also shown in the study by Campistol et al. (Campistol et al. 2005). Moreover, the combination of an inhibitor of purine synthesis (azathioprine or MMF) with glucocorticoids, CsA or sirolimus had no effect on bone loss (Campistol et al. 2005). Research has also demonstrated that the administration of low-dose CsA does not contribute to bone loss, which confirms the claim that bone resorption caused by immunosuppressive medication is dose-dependent and highlights the merit of combination low dose immunosuppressive therapy after transplantation (Goodman et al. 2001). In our study, mother rats receiving different combinations of immunosuppressive medication had significantly higher Ca levels in the groups given Tc, MMF and prednisone (group B2) and CsA, everolimus and prednisone (group B3). In group B1, the Ca level was also higher than that in the control, but the difference there was not significant. While statistical analysis failed to identify significant differences in Mg levels in mother rats, notably Mg levels across all the study groups were lower than in the control. The survey conducted by Sabbagh et al. showed that the use of CsA leads to the depletion of bone Mg and induces hypomagnesemia (Sabbagh et al. 2008). Hypomagnesemia may also be caused by prednisone and tacrolimus (Boncimino et al. 1999, Lote et al. 2000). Mg plays a prominent role in bone homeostasis (Launius et al. 2004). It influences the production and release of hormones responsible for regulating bone homeostasis and the sensitivity of receptors located on bone cells to those hormones. Mg deficiency has a negative effect on all phases of skeletal metabolism, decreasing both osteoblast and osteoclast activity, suppressing growth and bone formation

(Launius et al. 2004). On the other hand, hypomagnesemia is correlated with less pronounced post-transplantation bone loss, probably due to inhibiting PTH release (Boncimino et al. 1999). Mg levels in groups B21 and B11 were significantly higher. These were the groups exposed to prednisone, CsA and MMF (group B11 at half-dosage and group B21 at full-dosage). Thus, therapy based on prednisone, CsA and MMF seems to increase Mg levels in the bones of rat offspring.

Dental caries and periodontal diseases are some of the most common chronic conditions afflicting mankind (Sandoval et al. 2017). They have a negative impact on quality of life and constitute the principal reasons for tooth loss. With dental caries, teeth are damaged by bacteria fermenting simple sugars to organic acids. With periodontal diseases, the oral microflora regulate the host immune response, which results in the destruction of the tissues anchoring the teeth in the bone, that is the periodontium. Lifestyle (a diet rich in carbohydrates, smoking) as well as poor oral hygiene are the risk factors for these diseases (Sandoval et al. 2017). The release of bacterial metabolites into the bloodstream activates the inflammatory response and host immune response. In this way, infected teeth may add to the inflammatory burden in the body. This effect is further enhanced in patients undergoing immunosuppressive treatment, leading to dental caries, gingival bleeding and overgrowth, periodontitis, periapical lesions, abscesses, xerostomia and candidiasis (Wondimu et al. 2001, Sandoval et al. 2017). The deleterious effect on periodontal tissues may also be caused by reduced bone volume, reduced number of osteoblasts and increase of osteoclasts (Santos et al. 2012). Research has shown that glucocorticoids, cyclosporine, tacrolimus and sirolimus affect bone metabolism and tooth movement (Santos et al. 2012). Immunosuppressants also have a direct influence on tooth development and structure. Wondimu et al. observed that CsA and tacrolimus may lead to enamel defects and structural abnormalities (from opacity to hypoplasia) – Wondimu et al. (2001). On the other hand, as teeth are located in the dental bone, immunosuppressive drugs may penetrate into this tissue worse and the effect of immunosuppressive drugs in the teeth may be weaker compared to bone (Santos et al. 2012, Yadong et al. 2018). Our study demonstrated that the immunosuppressive regimens included in the analysis do not have a significant effect on the levels of Ca, P and Mg in the teeth of rats subjected to the immunosuppressive treatment and their offspring, except for group B13 (offspring of mothers exposed to CsA, prednisone and everolimus), wherein Mg levels were significantly lower than in the control group, which may be negative effects of immunosuppressive drugs.

In conclusion, the immunosuppressive regimens did not affect the levels of Mg and P in the rat model (mothers), but they did contribute to an increased bone Ca level. The immunosuppressive regimens administered at therapeutic doses are harmful to rat foetuses, as evidenced by the small number or lack of offspring in the tested groups (B1, B2, B3), most likely due to the inclusion of MMF and everolimus in the studied therapy regimens

(Shah, Verma 2016). The use of prednisone, CsA and MMF increases bone Mg levels in the offspring, while a therapy based on prednisone, CsA and everolimus significantly reduces the Mg level in the teeth of the offspring, potentially affecting mineralisation and strength of hard tissues. Due to the lack of similar works, it is not possible to directly compare the results. Additionally, authors usually study the effect of individual drugs on the mineralization of hard tissues, which is not always a reflection of the effect in the immunosuppressive regimens.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

All authors participated in the study design, interpretation, analysis, and review of the manuscript.

REFERENCES

Ahlström M., Pekkinen M., Lamberg-Allardt C. 2009. *Dexamethasone downregulates the expression of parathyroid hormone-related protein (PTHrP) in mesenchymal stem cells.* Steroids, 74: 277-282. https://doi.org/10.1016/j.steroids.2008.12.002

Aroldi A., Tarantino A., Montagnino G., Cesana B., Cocucci C., Ponticelli C. 1997. *Effects of three immunosuppressive regimens on vertebral bone density in renal transplant recipients: A Prospective Study*. Transplantation, 63: 380-386. https://doi.org/10.1097/00007890--199702150-00009

Bellorin-Font E., Rojas E., Carlini R.G., Suniaga O., Weisinger J.R. 2003. *Bone remodeling after renal transplantatio*n. Kidney Int., 63: S125-128. https://doi.org/10.1046/j.1523-1755.63.s85.30.x

Blaslov K., Katalinic L., Kes P., Spasovski G., Smalcelj R., Basic-Jukic N. 2014. *What is the impact of immunosuppressive treatment on the post-transplant renal osteopathy?* Int. Urol. Nephrol., 46: 1019-1024. https://doi.org/10.1007/s11255-013-0596-7

Boncimino K., Mcmahon D.J., Addesso V., Bilezikian J.P., Shane E. 1999. *Magnesium deficiency and bone loss after cardiac transplantation*. J. Bone Miner. Res., 14: 295-303. https://doi.org/10.1359/jbmr.1999.14.2.295

Campistol J.M., Holt D.W., Epstein S., Gioud-Paquet M., Rutault K., Burke J.T., Sirolimus European Renal Transplant Study Group. 2005. *Bone metabolism in renal transplant patients treated with cyclosporine or sirolimus.* Transplant Int., 18: 1028-1035. https://doi.org/10.1111/j.1432-2277.2005.00163.x

Coco M. 2003. *Prevention of bone loss in renal transplant recipients: A prospective, randomized trial of intravenous pamidronate*. J Am Soc Nephrol., 14: 2669-2676. https://doi.org/10.1097/01.ASN.0000087092.53894.80

Dounousi E., Leivaditis K., Eleftheriadis T., Liakopoulos V. 2015. *Osteoporosis after renal transplantation.* Int. Urol. Nephrol., 47: 503-511. https://doi.org/10.1007/s11255-014-0862-3

Goodman G.R., Dissanayake I.R., Sodam B.R., Gorodetsky E., Lu J., Ma Y.F., Jee W.S., Epstein S. 2001. *Immunosuppressant use without bone loss-implications for bone loss after transplantation.* J. Bone Miner. Res., 16: 72-78. https://doi.org/10.1359/jbmr.2001.16.1.72

Grotz W.H., Mundinger F.A., Rasenack J., Speidel L., Olschewski M., Exner V.M., Schollmeyer P.J. 1995. *Bone loss after kidney transplantation: a longitudinal study in 115 graft recipients.* Nephrol. Dial. Transplant., 10: 2096-2100.

Inoue T., Kawamura I., Matsuo M., Aketa M., Mabuchi M., Seki J., Goto T. 2000. *Lesser reduction in bone mineral density by the immunosuppressant, fk506, compared with cyclosporine in rats*. Transplantation, 70: 774-779. https://doi.org/10.1097/00007890-200009150-00011

Jolicoeur E.M., Qi S., Xu D., Dumont L., Daloze P., Chen H. 2003. *Combination therapy of mycophenolate mofetil and rapamycin in prevention of chronic renal allograft rejection in the rat*. Transplantation, 75: 54-59. https://doi.org/10.1097/00007890-200301150-00010

Kabat-Koperska J., Kolasa-Wołosiuk A., Wojciuk B., Wojciechowska-Koszko I., Roszkowska P., Krasnodębska-Szponder B., Paczkowska E., Safranow K., Gołembiewska E., Machaliński B., Ciechanowski K. 2016. *The influence of intrauterine exposure to immunosuppressive treatment on changes in the immune system in juvenile Wistar rats*. DDDT, 10: 2279-2288. https://doi.org/10.2147/DDDT.S102189

Kabat-Koperska J., Kolasa-Wołosiuk A., Pilutin A., Safranow K., Kosik-Bogacka D., Baranowska--Bosiacka I., Gołembiewska E., Kędzierska K., Domański L., Ciechanowski K. 2016. *Birth defects in juvenile Wistar rats after exposure to immunosuppressive drugs during pregnancy*. Histol. Histopathol., 2016: 43-55. https://doi.org/10.14670/HH-11-769

Kalantar-Zadeh K., Molnar M.Z., Kovesdy C.P., Mucsi I., Bunnapradist S. 2012. *Management of mineral and bone disorder after kidney transplantation*. Curr. Opinion Nephrol. Hyperten., 21: 389-403. https://doi.org/10.1097/MNH.0b013e3283546ee0

Katz I.A., Takizawa M., Jaffe I.I., Stein B., Fallon M.D., Epstein S. 1991. *Comparison of the effects of FK506 and cyclosporine on bone mineral metabolism in the rat. A pilot study*. Transplantation, 52: 571-574.

Kovacs C.S. 2015. *Calcium, phosphorus, and bone metabolism in the fetus and newborn*. Early Human Develop., 91: 623-628. https://doi.org/10.1016/j.earlhumdev.2015.08.007

Kurdián M., Herrero-Fresneda I., Lloberas N., Gimenez-Bonafe P., Coria V., Grande M.T., Boggia J., Malacrida L., Torras J., Arévalo M.A., González-Martínez F., López--Novoa J.M., Grinyó J., Noboa O. 2012. *Delayed mTOR Inhibition with low dose of everolimus reduces TGFβ expression, attenuates proteinuria and renal damage in the renal mass reduction model*. PLoS ONE, 7: e32516. https://doi.org/10.1371/journal.pone.0032516

Launius B.K., Brown P.A., Cush E.M., Mancini M.C. 2004. *Osteoporosis: The dynamic relationship between magnesium and bone mineral density in the heart transplant patient*. Crit. Care Nurs. Q., 27: 96-100.

Lote C.J., Thewles A., Wood J.A., Zafar T. 2000. *The hypomagnesaemic action of FK506: urinary excretion of magnesium and calcium and the role of parathyroid hormone*. Clin. Sci., 99: 285-292.

Ma Y., Kobayashi T., Kuzuya T., Liu D., Nagasaka T., Yokoyama I., Miwa Y., Morozumi K., Oikawa T., Uchida K., Nakao A. 2006. *Is absorption profile of cyclosporine really important for effective immunosuppression?* Biol. Pharm. Bull., 29: 336-342. https://doi.org/10.1248/bpb.29.336

Martinez-Palli G., Hirose R., Liu T., Xu F., Dang K., Feiner J., Serkova N.J., Niemann C.U. 2011. *Donor pre-treatment with everolimus or cyclosporine does not reduce ischaemia-reperfusion injury in a rat kidney transplant model*. Nephrol. Dial. Transpl., 26: 1813-1820. https://doi.org/10.1093/ndt/gfq646

Palacios C. 2006. *The role of nutrients in bone health, from A to Z*. Crit. Rev. Food Sci. Nut., 46: 621-628. https://doi.org/10.1080/10408390500466174

Patschan D., Loddenkemper K., Buttgereit F. 2001. *Molecular mechanisms of glucocorticoid--induced osteoporosis*. Bone, 29: 498-505. https://doi.org/10.1016/S8756-3282(01)00610-X

Piao S.G., Bae S.K., Lim S.W., Song J.-H., Chung B.H., Choi B.S., Yang C.W. 2012. *Drug interaction between cyclosporine and mTOR inhibitors in experimental model of chronic cyclosporine nephrotoxicity and pancreatic islet dysfunction*. Transplantation, 93: 383-389. https://doi.org/10.1097/TP.0b013e3182421604

Rubin M.R., Bilezikian J.P. 2002. *The role of parathyroid hormone in the pathogenesis of gluco-*

corticoid-induced osteoporosis: A re-examination of the evidence. J. Clin. Endocrinol. Met., 87: 4033-4041. https://doi.org/10.1210/jc.2002-012101

Sabbagh F., El Tawil Z., Lecerf F., Hulin A., Maurois P., Dartevelle P., Bac P., German--Fattal M. 2008. *Impact of cyclosporine A on magnesium homeostasis: clinical observation in lung transplant recipients and experimental study in Mice*. Transplantation, 86: 436-444. https://doi.org/10.1097/TP.0b013e31817fe069

Sagiroglu T., Sezer A., Torun N., Yalta T., Yagci M.A., Sagiroglu G., Copuroglu E. 2014. *Protective effect of everolimus on renal ischemia reperfusion injury in rats*. Saudi J. Kidney Dis. Transpl., 25: 294-302.

Sandoval M.J., Zekeridou A., Spyropoulou V., Courvoisier D., Mombelli A., McLin V., Giannopoulou C. 2017. *Oral health of pediatric liver transplant recipients*. Pediatr. Transplant, 21: e13019. https://doi.org/10.1111/petr.13019

Santos R.L. dos, Lacerda M.C.M., Gonçalves R.T., Martins M.A., Souza M.M.G. de. 2012. *Immunosuppressants: implications in orthodontics*. Dental Press J. Orthod., 17: 55-61. https://doi.org/10.1590/S2176-94512012000200012.

Shah S., Verma P. 2016. *Overview of pregnancy in renal transplant patients*. Int. J. Nephrol., 2016: 1-7. https://doi.org/10.1155/2016/4539342

Stempfle H.-U., Werner C., Siebert U., Assum T., Wehr U., Rambeck W.A., Meiser B., Theisen K., Gärtner R. 2002. *The role of tacrolimus (FK506)-based immunosuppression on bone mineral density and bone turnover after cardiac transplantation: a prospective, longitudinal, randomized, double-blind trial with calcitriol1*. Transplantation, 73: 547-552. https://doi.org/10.1097/00007890-200202270-00010

van Westrhenen R., Aten J., Hajji N., de Boer O.J., Kunne C., de Waart D.R., Krediet R.T. 2007. *Cyclosporin a induces peritoneal fibrosis and angiogenesis during chronic peritoneal exposure to a glucose-based, lactate-buffered dialysis solution in the rat*. Blood Purif., 25: 466-472. https://doi.org/10.1159/000112475

Vicente-Rodríguez G., Ezquerra J., Mesana M.I., Fernández-Alvira J.M., Rey-López J.P., Casajus J.A., et al. 2008. *Independent and combined effect of nutrition and exercise on bone mass development*. J. Bone Miner. Metab., 26: 416-424. https://doi.org/10.1007/s00774-007--0846-9

Viklický O., Müller V., Zou H., Szabó A., Vitko Š., Heemann U. 2001. *RAD reduces compensatory renal graft hypertrophy in a rat model of chronic rejection*. Transplantation Proc., 33: 2320-2321. https://doi.org/10.1016/S0041-1345(01)02008-5

Weinstein R.S. 2011. *Glucocorticoid-induced bone disease*. N. Engl. J. Med., 365: 62-70. https:// //doi.org/10.1056/NEJMcp1012926

Westenfeld R., Schlieper G., Woltje M., Gawlik A., Brandenburg V., Rutkowski P., Floege J., Jahnen-Dechent W., Ketteler M. 2011. *Impact of sirolimus, tacrolimus and mycophenolate mofetil on osteoclastogenesis-implications for post-transplantation bone disease*. Nephrol. Dialys. Transplant., 26: 4115-4123. https://doi.org/10.1093/ndt/gfr214

Wondimu B., Németh A., Modéer T. 2001. *Oral health in liver transplant children administered cyclosporin A or tacrolimus*. Int. J. Paediatr. Dent., 11: 424-429.

Yadong J., Seung K.C., Ahmed S.S., Kong C., Xiaoying L., Erfan D., Mary A.M., Michael W., Huakun X., Lobat T., Zhihong N., Didier A.D., Radi M. 2018. *Nanomagnetic-mediated drug delivery for the treatment of dental disease*. Nanomedicine, 14(3): 919-927. DOI: 10.1016/j. nano.2018.01.013

Yazawa K., Ishikawa T., Ichikawa Y., Shin J., Usui Y., Hanafusa T., Fukunishi T., Sakai R., Nagano S., Fujita N., Mizuno K. 1998. *Positive effects of kidney transplantation on bone mass*. Transplant. Proc., 30: 3031-3033. https://doi.org/10.1016/S0041-1345(98)00917-8

Zhang R. 2012. *Metabolic bone diseases in kidney transplant recipients*. WJN, 1: 127. https:// //doi.org/10.5527/wjn.v1.i5.127