1,25-Dihydroxycholecalciferol with low-calcium diet reduces acute rejection in rat lung allotransplantation

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Abstract

OBJECTIVES: The effect of 1,25-dihydroxycholecalciferol (calcitriol, vitamin D3) with a low-calcium diet on the acute lung allograft rejection in a rat unilateral left lung transplantation model was evaluated.

METHODS: Three transplantation groups were studied (n = 5, male Brown-Norway to Fischer F344, 235 ± 15 g body weight): calcitriol and low-calcium diet, low-calcium diet and normal diet. Calcitriol (4 μg/kg/day) was injected intraperitoneally for 5 days, starting from the day of transplantation. In addition, two non-transplantation groups were compared: (n = 3, Brown-Norway) to measure the level of cytokines, and Fischer F344 receiving calcitriol and a low-calcium diet to measure the serum calcium level. The recipients of transplantation were killed on Day 5 post-transplant. The contralateral right main bronchus and the pulmonary artery were occluded for 5 min and blood was drawn for the blood gas analysis, and the grafts were assessed for histology (International Society for Heart and Lung Transplantation 1996/rank scale). Lung levels of interleukin (IL)-2, IL-6, IL-12 and tumour necrosis factor-α (TNF-α) were assessed within the calcitriol and low-calcium diet, low-calcium diet and Brown-Norway groups. The serum calcium level was assessed in the Fischer F344 group. An analysis of variance with Tukey’s post hoc test was used to compare the arterial blood oxygen pressure and the lung cytokine expression between groups. A non-parametric Kruskal–Wallis test followed by the Siegel and Castellan test was used to compare the differences between the groups according to the lung graft rejection grading. Student’s paired t-test was used to compare the serum calcium level.

RESULTS: The arterial PaO2 was significantly higher in the calcitriol and the low-calcium diet groups when compared with low-calcium diet or normal diet groups (356 ± 72 mmHg; P < 0.05 vs other groups). The arterial and bronchial rejection observed in calcitriol and low-calcium diet group was significantly milder than in the low-calcium diet or normal diet groups (A1-2, B1-2; P < 0.05 vs other groups). IL-2 and IL-6 levels were significantly higher in low-calcium diet vs calcitriol and low-calcium diet and Brown-Norway groups. IL-12 and TNF-α did not differ among the groups. There was no significant difference in serum calcium level before and after the treatment in the Fischer F344 group.

CONCLUSIONS: Calcitriol with a low-calcium diet treatment improves lung function, reduces lung allograft acute rejection, decreases IL-2 and IL-6 allograft expression and does not change the serum calcium level significantly.

Keywords: Calcitriol • Cytokines • Lung transplantation • Rejection

INTRODUCTION

Lung transplantation is an established therapeutic option in the end-stage of pulmonary disease. However, acute rejection limits the short-term survival of lung transplant recipients. In addition, the high level of immunosuppression, for example, compared with kidney recipients, needed in these patients causes a number of unwanted side effects of the different drugs. Therefore, substances that may attenuate the side effects are needed.

1,25-Dihydroxycholecalciferol (1,25-dihydroxyvitamin D3, calcitriol) has been studied as an immunosuppressant in recent years. The profile of side effects of this substance seems to be more favourable than the drugs currently used as standard medication following organ transplantation. It has been shown in vivo and in vitro that calcitriol strongly influences the immune system [1]. A number of mechanisms have been described that may explain the immunosuppressive properties of calcitriol and its analogues. It shifts the differentiation of the Th0 lymphocytes towards Th2 and inhibits the generation of Th1 lymphocytes [2], increases the suppressor activity of CD8+ and CD4+ effectors function. Furthermore, calcitriol inhibits the immunoglobulin G2a production by B cells, reduces the major histocompatibility

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complex (MHC) II expression, prevents the development of cytotoxic effects and inhibits the differentiation and maturation of dendritic cells [3]. An increased expression of MHC class II antigens is observed during the acute rejection in lung allotransplantation in Mongrel dogs [4]. Calcitriol also induces apoptosis in peripheral mononuclear cells and inhibits pathological angiogenesis [5]. In this study, we evaluated the effect of calcitriol on the acute rejection in one major and one minor immunologically mismatched, rat lung transplant model—Brown-Norway to Fischer F344.

METHODS

Experimental groups

A model of allogeneic left lung allotransplantation in rats with one major and one minor MHC mismatch (donor: Brown-Norway; recipient: Fischer F344; obtained from RCC Ltd., Biotechnology & Animal Breeding Division, Fullinsdorf, Switzerland) was used. All the animals were male. Five animal groups were studied—three transplantation groups (n = 5, calcitriol and low-calcium diet, low-calcium diet and normal diet), and two non-transplantation groups (n = 3, Brown-Norway and Fischer F344). Recipients in the calcitriol and low-calcium diet group received 4 µg/kg/day calcitriol by intraperitoneal injection for 5 days post-transplant and were put on a low-calcium diet for 12 days (7 days before and 5 days after transplantation). Recipients in the low-calcium diet group received a low-calcium diet for 12 days (7 days before and 5 days after transplantation). The normal diet group recipients were put on a normal diet before and after transplantation. The Brown-Norway group imitating donors served as controls for the measurement of cytokines and consisted of male Brown-Norway rats. The Fischer F344 group imitating recipients served to measure the serum calcium level before and after calcitriol injection for 5 days and a low-calcium diet for 12 days treatment and consisted of male Fischer F344 rats.

A low-calcium diet was fed to the calcitriol and low-calcium diet group and the animals of the Fischer F344 group from Day 0 to Day 12 throughout the experiment. On Day 7, orthotopic single left lung transplantations were performed from Brown-Norway to Fischer F344 rats of the transplantation groups, using the cuff technique [6] for the vessel anastomoses and a conventional running over-and-over suture for the bronchial anastomosis. Calcitriol (4 µg/kg/day i.p.) was given to the calcitriol and low-calcium diet group and the Fischer F344 animals from Day 7 of the experiment and continued until the animals were killed. The low 0.02% calcium diet was provided by the University of Wisconsin, Madison, WI, USA. Calcitriol (Rocaltrol®) and 10 ml solution, 1 µg/ml was provided by Roche, Basel, Switzerland.

All the animals received care in compliance with the ‘Principles of Laboratory animal care’ NIH publication Vol. 25, No. 28 revised 1996. The protocol was approved by the local animal study committee.

Operative procedure and measurements

Donor. The animal was anaesthetized in a glass chamber by allowing it to breathe 2% Halothane (SIGMA®), intubated and anaesthesia was maintained with Halothane. A left thoracotomy in the fourth intercostal space was performed. The neurovascular clips were put onto the left PA and left pulmonary vein (PV). The left main native bronchus was ligated with a 6-0 polyfilament suture (SofSilk, USCC, USA) and was cut off. An incision was made in both the PV and PA. The vessels were flushed with a heparinized saline solution. The cuffs were inserted into the recipient’s vessels, and 6-0 polyfilament ligatures (SofSilk, USCC) were placed around the cuffs and tied. The native PA and PV were cut off beyond the anastomosis and the native lung was removed. A 9-0 Monosof (Tyco Healthcare, Wollerau, Switzerland) running over-and-over continuous suture was used for the bronchial anastomosis. The ventilation and then retrograde perfusion followed by the antegrade perfusion of the graft were restored by removing the clips from the left bronchus, PV and PA, respectively. A chest drain (24Gx3/4 infusion set, Terumo®, Belgium) was inserted into the left hemithorax and the thoracotomy was closed with four layers of continuous sutures (4/0 Prolene, Johnson and Johnson®). The thoracic drain was removed after the animal restored spontaneous breathing, and the animal was extubated.

Assessment

Arterial blood oxygen partial pressure. On Day 12, 5 days post-transplant, the animals from the transplantation groups were pre-anæsthetized in a glass chamber by allowing them to inhale 4% Halothane (SIGMA®). Thiopental (Pentothal, Abbott AG) at a dose of 50 mg/kg was injected intraperitoneally. Heparin (Liquemin, Roche Pharma, Switzerland) was administered through a peripheral vein (500 IU/kg). A tracheotomy was performed, and the animal was ventilated using a 14-gauge i.v. catheter (Inyect®, Madrid, Spain), with 100% oxygen, f = 100/min, tidal volume (TV) = 10 ml/kg, and by a Harvard Rodent Ventilator (model 683, Harvard Apparatus, South Natick, MA, USA). After cutting the inferior vena cava and the left appendix of the heart, a small silicon hose was inserted into the main pulmonary artery (PA) via an incision in the right ventricle. Both lungs were flushed with 20 ml of a low-potassium dextrane solution (Perfadex®, Medisan Pharmaceutics, Uppsala, Sweden) at a pressure of 20 cm H2O. The trachea was then tied at the end-inspiratory position. The heart-lung block was removed and the left lung was separated ex vivo from the heart and the right lung. Twenty-four gauge cuffs were placed around the PA and vein; the vessels were everted and tied onto the cuff and fastened with 8-0 monofilament suture (Surgipro, USSC, USA). The lung was stored at 10°C in an low potassium dextrane solution, until implantation.

Recipient. The recipient was anaesthetized in a glass chamber by allowing it to breathe 2% Halothane (SIGMA®), intubated and anaesthesia was maintained with Halothane. A left thoracotomy in the fourth intercostal space was performed. The neurovascular clips were put onto the left PA and left pulmonary vein (PV). The left main native bronchus was ligated with a 6-0 polyfilament suture (SofSilk, USCC, USA) and was cut off. An incision was made in both the PV and PA. The vessels were flushed with a heparinized saline solution. The cuffs were inserted into the recipient’s vessels, and 6-0 polyfilament ligatures (SofSilk, USCC) were placed around the cuffs and tied. The native PA and PV were cut off beyond the anastomosis and the native lung was removed. A 9-0 Monosof (Tyco Healthcare, Wollerau, Switzerland) running over-and-over continuous suture was used for the bronchial anastomosis. The ventilation and then retrograde perfusion followed by the antegrade perfusion of the graft were restored by removing the clips from the left bronchus, PV and PA, respectively. A chest drain (2Gx3/4 infusion set, Terumo®, Belgium) was inserted into the left hemithorax and the thoracotomy was closed with four layers of continuous sutures (4/0 Prolene, Johnson and Johnson®). The thoracic drain was removed after the animal restored spontaneous breathing, and the animal was extubated.
Subsequently, the inferior vena cava and left appendix of the heart were incised and a small silicon hose was inserted into the main PA via an incision in the right ventricle. The lungs were then flushed with 20 ml of 0.9% NaCl under a pressure of 20 cm of H2O. The trachea was ligated at the end-inspiratory position and the tracheotomy tube was removed. The heart-and-lung block was explanted and the sample of the left grafted lung was put in a 10% formalin solution (SIGMA®) for histological analysis, and the rest of the graft was snap-frozen in liquid nitrogen for cytokine analysis. In the Brown-Norway group, the left native lung was used for the analysis of cytokines.

Rejection grading. The histological assessment of the acute rejection of the transplantation groups was done by a trained lung pathologist in a blinded fashion according to the Working Formulation for the 1996 Classification of Pulmonary Allograft Rejection of the International Society for Heart and Lung Transplantation (ISHLT) [7].

Lung cytokine

Quantitative reverse transcription polymerase chain reaction. The cytokine levels were assessed in the lungs of the calcitriol and low-calcium diet, low-calcium diet and Brown-Norway groups. Total RNA was isolated from the frozen lung tissue using the SV Total RNA Isolation Kit (Promega, Zurich, Switzerland) and then 1 μg of RNA was reverse-transcribed into cDNA (OMiscript™, Qiagen). Transcript levels for cytokines were quantified using real-time quantitative polymerase chain reaction (AMB Prism 7700; Perkin Elmer Applied Biosystems, Foster City, CA, USA). Primers and probes for the cytokines were designed using the Primer Express Software (Applied Biosystems). 6-carboxyfluorescein and tetramethylrhodamine were used as reporter and quencher dyes, respectively, for labelling the probes. Cytokine levels were normalized to 18S rRNA using a reporter and quencher dyes, respectively, for labelling the probes. The serum calcium level was assessed in the non-transplantation Fischer F344 group. The animals were fed with a low-calcium diet for 12 days. On Day 0, 500 µl of full blood was drawn from the left external jugular vein, using a syringe (Pico™ 50 Radiometer). The calcium concentration was measured with a radiometer (ABL 700 Series). From Days 7 to 12, the rats received 4 µg/kg/day calcitriol i.p. On Day 12 (the day on which the animals were killed), another 500 µl of blood was drawn from the vena cava inferior for the calcium level measurement.

Statistics

Analysis of variance (ANOVA) with Tukey’s post hoc test was used to compare the arterial blood oxygen partial pressure and the lung cytokine expression between groups. Oxygen partial pressure and interleukin (IL)-6 expression were transformed logarithmically for this analysis to obtain normal distributions. A non-parametric Kruskal–Wallis test followed by Siegel and Castellan post hoc test was used to assess differences between the groups according to the lung graft rejection grading. Student’s paired t-test was used to compare the serum calcium level. STATISTICA PL, version 7.1, Statsoft, Cracow was used for the statistical analysis.

RESULTS

Characterization of experimental groups

In the three transplantation groups (n = 5, calcitriol and low-calcium diet, low-calcium diet, normal diet), the donor and recipient rats weighed 235 ± 15 g, with no statistical difference between the groups. The entire transplantation procedure (donor lung explantation, ex vivo preparation and implantation) took 126 ± 18 min. The warm ischaemic time was 24 ± 6 min, without any statistical difference between the groups. In the two non-transplantation groups (n = 3, Brown-Norway, Fischer F344), the rats were of the same weight. The operative procedures were done by the author personally, under a stereoscopic microscope (Olympus™, SZX12, Japan) with a magnification of 6–20 times. Three recipients of the transplantation groups died because of technical errors and additional transplantations were carried out to replace these animals.

Arterial blood oxygen partial pressure

The arterial blood oxygen partial pressure was measured in all three transplantation groups. The results are shown in Fig. 1. ANOVA revealed the statistically highly significant difference among the three transplantation groups (P < 0.000001). Inter-group comparisons between the calcitriol and low-calcium diet group (356 ± 72 mmHg) and the low-calcium diet group (46 ± 8.0 mmHg), as well as between the calcitriol and low-calcium diet and the normal diet groups (36 ± 10.9 mmHg) are statistically significant (P = 0.00019 for each comparison, Tukey’s post hoc test) in contrast to the comparison between the low-calcium diet and normal diet groups (P = 0.49).

Rejection grading

The histological assessment was done in all three transplantation groups. The results are shown in Table 2. It demonstrates the significant difference between the calcitriol and low-calcium diet vs normal diet group (P = 0.005 and P = 0.008 for peri-arterial A

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### Table 1: Cytokines primer/probe sequence

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>Forward: 5’-CCAGAGTGTCAATCAACGC-3’ Reverse: 5’-GCGGAGTGGTGGTTTGGAG-3’ Probe: 5’-ACTCTCCTTCTTGCCACCCCTGT-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward: 5’-GAAAGAAGCTACAACTCATCG-3’ Reverse: 5’-AGGGAATCTGGCTGAAGCT-3’ Probe: 5’-TACGGAACAGCTATGAAGTTCCTCCGCA-3’</td>
</tr>
<tr>
<td>IL-12</td>
<td>Forward: 5’-CTGGACCTGGCAAAGTGCTTCAAC-3’ Reverse: 5’-TGCGCCTGCCACTCATGT-3’ Probe: 5’-CCCAAACACCTGGTGAAACCGGAC-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: 5’-ACAAGGCTGCCCCGCACAC-3’ Reverse: 5’-TCTCTGATGAAGTGCAAAAC-3’ Probe: 5’-TGCTTCACACCACGGTGCA-3’</td>
</tr>
</tbody>
</table>
Lung cytokine expression

The cytokine levels were measured within the calcitriol and low-calcium diet, low-calcium diet and Brown-Norway groups. The expression of the IL-2 and IL-6 cytokines, as shown in Fig. 4, was the strongest in the low-calcium diet group and the lowest in the Brown-Norway group. ANOVA showed significant differences between groups for both IL-2 (P = 0.0051) and IL-6 (P = 0.000022). According to Tukey’s test, statistical significance was reached in the low-calcium diet against the calcitriol and low-calcium diet group (P = 0.026 for IL-2 and P = 0.019 for IL-6) or against Brown-Norway (P = 0.021 and P = 0.00019, respectively), but the difference in calcitriol and a low-calcium diet against the Brown-Norway group was significant only for IL-6 (P = 0.00070) and not for IL-2 (P = 0.92).

ANOVA showed no significant intergroup differences for IL-12 (P = 0.68) nor tumour necrosis factor-α (TNF-α; P = 0.29).

DISCUSSION

In this study, we have shown that 1,25-dihydroxycholecalciferol (calcitriol, vitamin D3) reduces acute lung allograft rejection. This is illustrated by better graft function (higher arterial blood oxygen pressure) and less histological damage (lower ISHLT 1996 rejection grading) in the calcitriol-treated recipients of the calcitriol and low-calcium diet group. The high dose of calcitriol administration combined with a low-calcium diet did not increase serum calcium levels in the Fischer F344 group. We did not create a control group receiving calcitriol and a normal diet, as we were afraid of hypercalciuria, hypercalcaemia and its sequels, such as calcium deposition in soft tissue. To our surprise, we did not register any changes in the calcium level and in future, a control group receiving calcitriol and a normal diet...
could be used. We have also shown that calcitriol-treated, less-rejected grafts express lower level of IL-2 and IL-6, but the IL-12 and TNF-α level remains uninfluenced.

Calcitriol is the active form of Vitamin D—cholecalciferol. In mammals, cholecalciferol is hydroxylated on C-25 in the liver and on C-1 in the kidneys. Until the late 70s, calcitriol was considered to be a regulator of calcium, phosphorus and bone metabolism only. In the 80s, the immunoregulatory properties of calcitriol have been discovered, and it has been shown that calcitriol suppresses T-helper-cell-mediated delayed hypersensitivity. The immunosuppressive properties of calcitriol are mediated by cells possessing nuclear Vitamin D receptor (VDR) [8]. The VDR is present in the cell cytoplasm and binds to the DR-3 promoter region of target genes to stimulate or suppress transcription, followed by translation of characteristic proteins. VDR changes its three-dimensional structure after specific, i.e. calcitriol activation. This is the key element of its action in the cell nucleus. Many factors influence the DNA transcription into mRNA—for example, VDR homo- or heterodimerization with retinoid X receptor. This means that the VDR complex may enter the nucleus and bind to its DNA. A lot of proteins may bind to the VDR complex which increase or decrease its chromatin condensation, thus acting as a co-repressor or co-activator and at the same time increasing or decreasing the target gene transcription. The co-activators bind the few additional proteins, building connections in the basic transcription mechanism. Therefore, little changes in the three-dimensional VDR shape caused by the activator may lead to a modulation in the protein binding and gene transactivation. This feature of nuclear receptors makes them an interesting therapeutic goal [9]. Hundreds of genes reacting with the elements of the nuclear VDR have a direct or indirect impact on the cell cycle, proliferation, differentiation or apoptosis. Apart from calcium regulation, Vitamin D influences physiologic and pathologic cell growth, carcinogenesis, immunologic and coronary vessel function [10]. VDR is a very common protein and is present in nearly every cell of the
mammals. Almost 3% of the human genome can be influenced by the VDR [11]. VDRs are present not only in the intestine, bone and kidney, but also in islet cells of the pancreas, parathyroid gland, haematopoetic cells, promyelocytes, activated lymphocytes, T-cells of thymus, keratinocytes of reproductive organs and skin. It has been proved that calcitriol inhibits the apoptosis of hepatocytes after allogeneic rat liver transplantation [12].

Calcitriol promotes Th0 differentiation into Th2 and, at the same time, inhibits differentiation into the Th1 subpopulation. That decreases the level of Th1-produced cytokines IL-2 and TNF-α. The lower concentration of these two cytokines decreases the MHC class II antigen expression on the antigen presenting cell (APC) surface. Calcitriol inhibits the production of IL-12, which is among the stimuli responsible for the activation of natural killer (NK) cells. Further research in the field of autoimmune diseases and organ transplantation were very promising. Experimental allergic encephalomyelitis in a murine model of immune diseases and organ transplantation were very promising. Experimental allergic encephalomyelitis in a murine model of multiple sclerosis has been totally or partially prevented, as well as lupus nephritis in mice, Heymann's nephritis [13], experimental autoimmune thyroiditis and an experimental model of human arthritis. Furthermore, acute rejection was reduced after the transplantation of pancreatic islets grafts in non-obese type I diabetes mice, and the mean survival time of non-vascularized neonatal heart transplants in rats and mice was prolonged. The VDR agonists can help us to treat ischaemic heart disease, as there is a higher risk of developing this condition by the patients with a lower serum level of Vitamin D or calcitriol [14].

The anti-proliferative effect of calcitriol on skin keratinocytes has been used in the treatment of dermatological disorders, e.g. psoriasis. Calcitriol stimulates the cell to differentiate into the final form and then to stop further proliferation (e.g. promyelocytes into monocytes). In other words, it causes the terminal differentiation in cells possessing VDR.

Calcitriol is an agonist of VDR and influences dendritic cells possessing the function of APCs. Activated VDR induces immunological tolerance in the APC and causes its maturation, though there are papers stating opposite, claiming that calcitriol inhibits APC maturation and differentiation, decreasing the expression of co-stimulating particles [15]. Calcitriol influences the monocytes (dendritic cells and macrophages precursors), which activate CD4+ lymphocytes in such a way that the interferon-γ level is decreased and IL-10 level is increased [16].

In allotransplantation, NK cells recognize the decrease of MHC antigens expression and mediate the target cell death by perforin, TNF-β, granzymes and by antibody-dependent cell-mediated cytotoxicity. On the other hand, IL-2 activates CD8+ T lymphocytes, which show cytotoxicity in the context of class I MHC antigens. Calcitriol influences not only the T-cell lymphokine production, but also T-cell proliferation, as it arrests the cells in the G0/G1 transition phase. Calcitriol also blocks IL-2 transcription mediated by nuclear factor of activated T cells and interacts with the T-cell activation cascade at a level downstream calcineurin [17].

In this study, the graft function of the calcitriol and low-calcium diet group was highly improved in comparison with the untreated controls of the low-calcium and normal diet groups. These results were correlated with the histologicalal analysis showing much higher rejection in control grafts of the low-calcium and normal diet groups.

In our experiment, four key cytokines were chosen to evaluate the effect of calcitriol. In this study, we were able to show reduced IL-2, but not IL-12 expression. IL-6 has been shown to be the lung ischaemia-reperfusion injury [18] and kidney acute rejection marker [19], and IL-2 was found to be an acute rejection marker in the heart transplant [20]. In our study, we confirmed that the highest expression of IL-6 was observed in the severely rejected grafts (low-calcium diet group), reduced IL-6 was noted in calcitriol-treated grafts (calcitriol and low-calcium diet group) and significantly lower IL-6 was found in normal lung tissue (Brown-Norway group). IL-6 was the most sensitive cytokine differentiating significantly all three assessed groups from each other. TNF-α and IL-12 expression, however, was not altered by calcitriol and low-calcium diet treatment. The cytokine expression was measured in the lung tissue homogenate, though it would also be interesting to check it in the bronchoalveolar lavage fluid. We did not perform it in order not to blur the lung tissue homogenate measurements. We think that either of these two could be evaluated in one recipient, but not both simultaneously.

Nowadays, to diagnose lung allograft acute rejection, the lung tissue sample has to be obtained or serum cytokine level has to be assessed. But it is also possible to suspect acute cellular rejection by measuring the uric acid serum level, as it was shown in the heart transplantation rejection by patients [21] and this can be used for rejection monitoring purposes in future.

In conclusion, calcitriol can be considered as a new experimental immunosuppressant, improving graft function markedly. Calcitriol inhibits the expression of IL-2, and it is consistent with the results of other studies [22], where acute rejection of the rat allografted lung increases the IL-2 mRNA level in the lung tissue. 1,25-Dihydroxycholecalciferol inhibits the expression of IL-6, but does not influence IL-12 and TNF-α level in lung allografts. The lack of acute rejection influencing TNF-α level was observed by others [23]. The calcitriol dose in the present experiment was high (4 μg/kg/day) and we have previously proved that 0.5 μg/kg/day was a sub-therapeutic dose and does not affect acute rejection alone [24].

There are some limitations in this study. The serum calcium level could not be measured in the transplantation groups, as the blood for the partial oxygen pressure measurement has to be heparinized and it makes the calcium assessment improper. Also, the rat blood sample size limits the number of assessments possible. In future, the experiment could therefore be repeated on the bigger animal model. In this study, we have used the older version of ISHLT rejection grading. In the new issue coming from 2007 [25], previous B1 and B2 stages are combined into B1R and B3 and B4 into B2R. For statistical reasons, it was easier to use the older, more stratified classification.

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Conflict of interest: none declared.
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