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Asymmetric Dimethylarginine and Endothelial Progenitor Cells After Renal Transplantation: the Effect of Exercise Training

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Summary

Level of asymmetric dimethylarginine (ADMA) is elevated and endothelial progenitor cells (EPC) and stem cells (SC) are decreased in patients undergoing renal transplantation (Tx) and may contribute to cardiovascular complications. We tested the hypothesis that ADMA, EPC and SC can be influenced with regular physical exercise early after Tx. Blood samples of ADMA, EPC, SC, adipocytokines and metabolic parameters were randomly obtained from 50 transplant patients before and 6 months after exercise program (Group I). Fifty age, sex, HLA typing, duration of dialysis and immunosupression regimen-matched non exercising transplant were examined as controls (Group II). After 6 months, in Group I ADMA decreased (3.50±0.45 vs 2.11±0.35 μmol/l, $P<0.01$) and was lower comparing to Group II ($P<0.01$), SC and EPC also decreased (2816±600 vs 2071±480 cells/ml resp. 194±87 to 125±67 cells/ml, $P<0.02$). Next changes in Group I: adiponectin ($P<0.01$), leptin ($P<0.01$), resistin ($P<0.02$). Visfatin, blood lipids, HbA1c, insulin and blood pressure were also influenced by training program ($P<0.05$).

Key words

Renal Transplantation • ADMA • EPC • Physical Exercise • Adipocytokines

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Introduction

Patients after renal transplantation face a particularly high risk of cardiovascular disease and mortality. Vascular dysfunction may be linked to reduced nitric oxide (NO) bioactivity and increased circulating concentrations of the endogenous NO synthase inhibitor asymmetric dimethyl L-arginine (ADMA) as a demonstrable marker of endothelial dysfunction in renal-disease patients (Kielstein *et al.* 2002).

Renal transplant patients have endothelial dysfunction and higher ADMA concentrations than healthy persons, which might contribute to higher cardiovascular morbidity in this population (De Matos *et al.* 2006). After successful renal transplantation, the level of ADMA decreases with improved endothelial function (Yilmaz *et al.* 2005). Cardiovascular complications in kidney transplant recipients can be related also to reduced circulating endothelial progenitor cells (EPC) and stem cells (SC). EPC and SC are derived from bone marrow, mature into endothelium and are crucial in endothelial recovery after ischemic injury (Ulbich *et al.* 2004, Steiner *et al.* 2006). Stimulatory and inhibitory factors modulate EPC and SC levels and function (e.g. age, hypertension, diabetes, obesity and hyperlipidemia). EPC and SC

function improves after kidney transplantation with normalisation of renal function (Di Marco *et al.* 2011).

A major factor potentially affecting the presence in circulating EPC and SC is aerobic exercise training. Its beneficial effect may be explained by the interplay of NO up/down regulation, and influence of proinflammatory cytokines in tissue.

Regular physical training has been shown to improve endothelium-dependent vasodilation and the cardiovascular risk profile in patients with ischemic heart disease (Werner *et al.* 2005, Umemura *et al.* 2008). It can influence cardiovascular risk factors in diabetics and chronic renal disease (Mittermayer *et al.* 2005, Schlager *et al.* 2011), but the effects of regular exercise on EPC, SC and ADMA, a novel cardiovascular risk markers in renal transplant patients, are not yet completely elucidated.

Materials and Methods

This study was approved by the Human Ethics Review Committee of the Institute for Clinical and Experimental Medicine (Prague, Czech Republic), and complies with the Declaration of Helsinki, including current revisions and the Good Clinical Practice Guidelines. The procedures followed were in accordance with institutional guidelines. All subjects gave written, informed consent before enrollment in the study.

Study subjects

In this prospective randomized study, 50 patients (27 male and 23 female; mean age 59±7.3 years; range 25 to 71 years; median age 54.0 years) who underwent a first cadaveric renal transplantation at the Transplant Center Institute for Clinical and Experimental Medicine (Prague, Czech Republic) since January 1, 2012 and who agreed to participate in a supervised aerobic exercise program for six months were studied.

The control group consisted of 50 (27 male and 23 female) transplant patients matched in age, sex, HLA typing, duration of previous dialysis, history of cardiovascular disease and immunosupression regimen who did not exercise regularly and did not participate in the training program.

The collection of patient data was completed by December 31, 2013. Patients were examined by the Clearance Laboratory of the Division of Metabolism at the Department of Nephrology (Transplant Center, Institute for Clinical and Experimental Medicine, Prague, Czech Republic) up to 6 months after renal transplantation. Blood samples were drawn two weeks after surgery and after 6 months. Renal function was examined using inulin and creatinine clearances.

The long-term, triple-drug immunosuppression protocol included regime based on FK 506-tacrolimus (Prograf, Astellas, Prague, Czech Republic), mycophenolate mofetil (Cellcept, Hoffmann-La Roche, Basel, Switzerland), 1 to 2 g/day, and steroids up to 10 mg/day (Prednisone, Zentiva, Prague, Czech Republic).

Four of 50 exercising patients and three of 50 non exercising patients suffered from diabetes mellitus and were treated with insulin. There were no acute infections at time of the examination (4 weeks before blood sampling).

Patients with a history of cardiovascular events were excluded, and the patients were nonsmokers. All patients were regularly treated with angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and statins, based on blood-pressure control and laboratory examination.

Evaluation of physical condition and regular exercise regimen

Physical condition was evaluated with "Senior Fitness Test-SFT" and HRQOL with standardized questionnaire SF36-Bref. Firstly, we compared results with population norms and secondly after an exercise program (pre-post-tests).

The patients after renal transplantation in exercising group participated in a 6-month training program of stationary cycling. Each exercise session lasted for 1 h and workloads were slowly increased on an individual basis. Following a 3-5 minute warm-up, resistance was increased until a heart rate of 60-70 % of the previously established difference from resting to maximum heart rate was achieved. This workload was maintained over 40 min, followed by a 5 min cool-down. During the first two weeks, training took place twice a week, and during the remaining study period, three times a week. All training was guided and supervised by a physician. The patients were allowed to carry out an additional training program at home, but compliance with the supervised training sessions had to be >60 % for study eligibility. The intervention part includes a 2-week adaptation phase, a 10-week development phase, and a 12-week maintenance phase (already in the home setting), complemented by assessment performed by

a specialist in kinetic anthropology (Teplan *et al*. 2012). The non-exercising transplant controls did not participate in a training program.

Anthropometric examination, sampling, and clearance methods

Anthropometric examination of patients was performed at basal state, at the beginning and end of the study. All subjects were measured and weighed, and their BMI was calculated. Renal function was estimated in the Clearance Laboratory of the Department of Nephrology (Transplant Center, Institute for Clinical and Experimental Medicine, Prague, Czech Republic) using an inulin clearance technique (Cin) and corrected creatinine clearance (Ccr) technique. A precise measurement of Cin requires the establishment of a balance state of plasma inulin concentration for the urine-collection period. To secure a sufficiently large urine volume, patients drank 0.5 to 1.0 liter of water, 1 h before the investigation began. The renal clearance of inulin was measured with a plasma level of inulin between 20 to 30 mg%; this was achieved by an initial load intravenously and a subsequent maintenance infusion. With a longer (60 min) urine collection period, a blood sample was collected at the beginning, in the middle, and at the end of the period. Creatinine clearance measurement involved a precise urine collection over 24 h with 2 venous blood samples at the start and end of the collection period.

Blood chemistry

Blood samples were collected into evacuated tubes with ethylenediaminetetraacetic acid, and the blood was immediately centrifuged. All patients fasted for at least 10 h before sampling, to avoid the influence of methionine from food on ADMA concentration. For ADMA quantification, an enzyme-linked immunoassay method (Kit ADMA, ELISA, DLD Diagnostika GmbH, Hamburg, Germany) and an AUTO-EIA II microplate reader (Labsystems Oy, Espoo, Finland) were used. This competitive method uses the microtiter plate format. The ADMA is bound to the solid phase, and ADMA in samples is acylated and competes with the bound ADMA for a fixed number of rabbit anti-ADMA antiserum binding sites. Afterward, equilibrium free antigen and free antigen-antiserum complexes are removed by washing. The bound antibody is detected by anti-rabbit peroxidase and 3.30, 5.50-tetramethylbenzidine as a peroxidase substrate. The final product of this reaction is monitored at 450 nm. The amount of antibody is inversely proportional to the ADMA concentration of sample.

Detection of endothelial progenitor cells (EPC) and stem cells (SC) in peripheral blood will be performed using determination of surface antigen expression. Prior to staining with a specific monoclonal antibody, 200 µl of peripheral blood will be incubated with 40 µl of fetal serum. Next, monoclonal antibody will be added to samples, i.e. 40 µl of antiCD 34 conjugated with phycoerythrin (PE) (Beckman Coulter), 20 µl antiCD 45 conjugated with fluorescein isothiocyanate (FITC) (Beckman Coulter) and 10 µl anti-KDR conjugated with Alexa Fluor 647 (e-Bioscience). Analyses are to be performed using an autolyzer (CyAn, Beckman Coulter), with each analysis assessing the expression of the monitored surface antigens per one million cells of peripheral blood. Based on human studies, stem cells will be defined as mononuclear $CD34^{+}/CD45^{low+}$ cells and EPC as mononuclear $CD34^{+}/CD45^{low+}/KDR^{+}$ cells.

After an overnight fast, venous blood samples were drawn and promptly centrifuged, and the plasma was stored at 22 °C until an adiponectin assay was performed. Plasma ADPN concentrations were measured in duplicate by radioimmunoassay (Human Adiponectin RIA Kit, Dinco Research, Inc., St. Charles, MO, USA). The ADNP levels of Group I (exercising renal transplant patients) and Group II (non exercising renal transplant patients) were measured at the same time. Serum leptin was measured by a commercial enzyme-linked immunoassay kit (Bio-Vendor, Prague, Czech Republic).

Serum visfatin levels were measured by enzyme immunoassay (ELISA) according to the manufacturer's protocol (BioVision Research Products, Mountain View, USA).

Inulin (polyfructosane S) was analyzed using anthrone on a spectrophotometer at wavelength 580 nm (Antelie Light Secoman, Domond Cedex, France).

Total cholesterol, HDL-cholesterol and triglycerides were determined using an enzymatic colorimetric method with an Olympus AU 600 analyzer and reagents from Olympus Diagnostics, GmbH (Hamburg, Germany). LDL cholesterol was calculated using Friedewald's formula. Serum insulin concentrations were measured using a commercial RIA kit (CisBio International, Lyon, France); glycated hemoglobin (HbA1c) was analyzed using liquid chromatography on a Tosoh HLC-723 G7 (Shiba, Minato-Ku, Tokio, Japan).

Statistical analysis

SigmaStat software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. A *t*-test or Mann-Whitney rank-sum test was used to compare data from the two groups of Tx patients. The relations between respective variables were assessed using Pearson's or Spearman's correlation coefficient as appropriate. Results were considered statistically significant at $P<0.05$.

Table 1. Clinical and biochemical characteristics of exercising and non exercising transplant subjects. Exercising kidney transplant subjects in the first month after transplantation before (Group IB) and 6 months after training (Group IA). Control group of kidney transplant non exercising subjects in the first month after transplantation (Group IIB) and after six months of follow-up (Group IIA).

Parameter	Group I_B (exercise before)	Group II_B (no exercise before)	Group I_A (exercise after)	Group II_A (no exercise after)	Statistical significance Gr I_B vs I_A	Statistical significance Gr I_A vs II_A
Number of patients	50	50	50	50	NS	NS
Gender (M/F)	27/23	27/23	27/23	27/23	NS	NS
Age (years)	59 ± 7.3	57 ± 8.2	59 ± 7.3	57 ± 8.2	NS	NS
BMI (kg/m^2)	24.4 ± 3.3	24.0 ± 2.6	27.2 ± 4.2	30.0 ± 3.1	$p < 0.05$	$p < 0.02$
C_{in} (ml/min/1.73 m^2)	28.6 ± 10.2	30.2 ± 8.4	60.1 ± 8.7	58.2 ± 8.0	$p < 0.01$	NS
C_{cr} (ml/min/1.73 m^2)	32.4 ± 7.4	34.4 ± 6.5	74.2 ± 6.6	68.4 ± 7.4	$p < 0.01$	NS
ADMA (μmol/l)	3.50 ± 0.45	3.30 ± 0.52	2.11 ± 0.35	3.25 ± 0.34	$p < 0.01$	$p < 0.01$
SC (cells/ml)	2816 ± 600	2720 ± 560	2071 ± 480	2580 ± 520	$p < 0.02$	$p < 0.02$
EPC (cells/ml)	194 ± 87	206 ± 72	125 ± 67	184 ± 68	$p < 0.02$	$p < 0.05$
ADPN (μg/ml)	15.4 ± 6.6	12.1 ± 7.8	22.3 ± 6.2	17.6 ± 4.4	$p < 0.01$	$p < 0.01$
Leptin (ng/l)	51.3 ± 11.2	58.3 ± 12.4	20.3 ± 9.2	45.4 ± 10.2	$p < 0.01$	$p < 0.01$
Resistin (μg/ml)	20.8 ± 10.1	18.2 ± 12.1	14.6 ± 6.4	18.4 ± 6.6	$p < 0.02$	NS
Visfatin (ng/ml)	1.8 ± 0.2	2.0 ± 0.3	1.2 ± 0.1	1.6 ± 0.4	$p < 0.05$	NS
Cholesterol (mmol/l)	6.1 ± 2.1	5.8 ± 1.6	5.4 ± 2.2	5.9 ± 0.4	$p < 0.05$	NS
LDL-cholesterol (mmol/l)	3.9 ± 1.1	3.6 ± 1.6	2.6 ± 1.9	3.5 ± 1.2	$p < 0.05$	NS
Triglycerides (mmol/l)	3.8 ± 1.6	3.5 ± 1.3	2.8 ± 1.0	3.4 ± 1.2	$p < 0.05$	NS
HbA_{1C} (%)	5.3 ± 1.4	4.8 ± 1.2	4.2 ± 1.2	5.0 ± 1.3	$p < 0.05$	NS
Insulin (pg/ml)	365.3 ± 40.1	242 ± 30.1	292.4 ± 49.3	320.6 ± 56.4	$p < 0.05$	NS
Systolic BP (mm Hg)	138 ± 12	142 ± 8	135 ± 7	138 ± 10	$p < 0.05$	NS
Diastolic BP (mm Hg)	90 ± 10	94 ± 7	90 ± 6	87 ± 7	$p < 0.05$	NS

Values are means ± SEMs. Statistical significance is from unpaired t-test or Mann-Whitney rank-sum test. NS – non significant, Statistical significance Group I_B vs Group II_B all NS. BMI – body mass index, C_{in} – inulin clearance, C_{cr} – creatinine clearance, ADPN – adiponectin, HbA1c – glycated Hb, ADMA – asymmetric dimethylarginine, EPC – endothelial.

Results

Basic clinical characteristics of both transplant patients groups are listed in Table 1.

They did not significantly differ with respect to age, gender or renal function measured by inulin and creatinine clearances before and after examination. There were no significant differences in ADMA levels, EPC and SC counts between both groups before the training program began. After six months of exercise, ADMA level in the Group I decreased (3.50±0.45 vs 2.11±0.35 μmol/l, P<0.01) and was also lower comparing

to Group II (2.11±0.23 vs 3.25±0.35 μmol/l, P< 0.01). In the same period, SC and EPC also decreased (2816±600 vs 2071±480 cells/ml resp. 194±87 to 125±67 cells/ml, P<0.02) in Group I, but in Group II changes were non significant (P=0.11). Next changes were found in Group I after six months of exercising: adiponectin (ADPN) (15.4±6.6 vs 22.3±6.2 mg/ml, P<0.01), leptin (51.3±11.2 vs 20.3±9.2 ng/l, P<0.01), resistin (20.8±10.1 vs 14.6± 6.4 mg/ml, P<0.02), and visfatin (1.8±0.2 vs 1.2±0.1, P<0.05). Patients in Group II revealed significant increase in BMI (P<0.01).

With respect to lipid metabolism parameters, there were small yet significant decreases in cholesterol, LDL-cholesterol, and triglycerides levels in Group I (P<0.05), and also decrease in HbA1c, plasma insulin and blood pressure (P<0.05). Renal function measured by inulin and creatinine clearance was normalized (P<0.01).

Discussion

Patients after renal transplantation have highly increased cardiovascular comorbidity compared with healthy persons and cardiovascular complications are the leading cause of death of kidney transplant recipients. Their etiology could be related to endothelial dysfunction associated with reduced counts of circulating endothelial progenitor cells (EPC) and stem cells (SC) (Allegra *et al*. 2009). Vascular dysfunction may also be linked to reduced nitric oxide (NO) bioactivity and increased circulating concentrations of the endogenous NO synthase inhibitor asymmetric dimethyl L-arginine (ADMA) as a demonstrable marker of endothelial dysfunction in renal-disease patients. Circulating progenitor cells have also been studied in kidney transplant recipients. While resumption of renal function and normalization of metabolic disorders related to chronic renal failure results in a mild rise in EPC counts in peripheral blood, the levels are still below those seen in healthy individuals (Werner *et al.* 2005, Herbrig *et al.* 2006a, Steiner *et al.* 2006, Tongers *et al.* 2007, Umemura *et al.* 2008, Chhabra *et al.* 2009). A major factor potentially affecting the presence in circulating progenitor cells in the plasma is aerobic exercise training. In patients, regular exercise affects not only muscular strength and muscular structure thus enhancing independence in mobility, it can also significantly improve a number of metabolic disorders (diabetes, dyslipidemia, bone disease, renal anemia, hormonal homeostasis, etc.). Recently, there have been studies documenting the beneficial effect of regular physical activity on ECP count after an exercise session also in renal patients (de Groot *et al.* 2005, Soler *et al.* 2005, Herbrig *et al.* 2006b). Regular physical activity is also believed to improve ones cardiovascular prognosis and may affect the EPC count and their functionality (Cheema *et al.* 2005). The improvement may be partly explained by bone marrow stimulation as a result of locally improved NO availability and normalisation of renal function (Steiner *et al.* 2006). In our study we found significant decrease of EPC and SC after six months of exercising in Group I. We suppose, the effect of exercising on EPC and SC could be in patients after renal transplantation modulated by long-term immunosupression drugs (cyslosporin or tacrolimus) which can activate stimulatory or inhibitory factors in bone marrow, but exact mechanism is still unknown (Riegersperger *et al.* 2013).

As shown by studies of morbid obesity, increased pro-inflammatory adipocytokine activity may result in a decrease in circulating EPC and SC counts before diet-induced loss of visceral adipose tissue (Juskowa *et al.* 2006, Johansen 2007) The role of increased BMI confirmed in Group II (non exercising), but suprisingly also in Group I (exercising) transplant patients after six months of follow-up can lead to decreased effect of aerobic exercising program on EPC and SC.

The raised ADMA levels in our study cohort might represent a marker or mediator of reduced basal NO production which could be improved by aerobic exercise. This study demonstrates that elevated ADMA concentrations decreased in patients after kidney transplantation during a regular program of aerobic exercise for 6 months; however, the effect of training on ADMA could be transient and could be abolished after termination of a physical exercise program (Teplan *et al.* 2008).

Only slight improvement of glycemic control is consistent with previous results showing that physical activity does not automatically improve glycemic control in patients after renal transplantation in the absence of a controlled diet. Blood lipids mildly decreased (P<0.05) and but an association with ADMA was not found.

After successful kidney transplantation adiponectin levels can slightly decrease, and suggested that kidneys participate in the biodegradation and elimination of adiponectin. However, there was no correlation between alteration of renal function and the

decrement of plasma adiponectin levels in the study. In leptin, we confirmed previous data showing an increased level of plasma leptin in renal transplant patients.

Resistin was originally discovered as an adipocyte-derived hormone whose levels increased in obesity, and it was suggested to link obesity to insulin resistence. Here we show for the first time that resistin behaves similarly to other adipokines. Visfatin is ubiquitously expressed in many tissues and was recently demonstrated to be an adipokine that is up regulated in visceral fat cells. Visfatin exerts insulin mimetic effects and, in addition to energy metabolism, plays a role in innate immunity and inflammation.

The limitations of our study are the lack of a chronic renal disease single kidney control patients without immunosuppressive therapy in exercising and non exercising regimen and analysis of long-term effect of exercising.

In conclusion, ADMA level, EPC and SC counts, selected adipokines and metabolit changes were influenced by early regular excercise in patients after kidney transplantation. The potential role of long-term immunosupression drugs and post-transplant obesity should be more precisely analyzed. This regimen may be helpful in decrease of cardiovascular risk after kidney transplantation and needs to be confirmed in long-term studies with cardiovascular end points.

Conflict of Interest

There is no conflict of interest.

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