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Increased Graft Survival through Ectonucleotidases Modulation in Platelets and Lymphocytes of Kidney Transplanted Patients

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Authors' contributions

This work was carried out in collaboration among all authors. Conceptualization authors AM, MDB and DTRS. Methodology authors AM, MVGR and AP. Formal analysis and investigation author AM. Writting – preparation of the original draft authors AM, SFVOM, MVGR and MRB. Writting – profreading and editing authors SFVOM, MRB and DTRS. All authors read and approved the final manuscript.

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ABSTRACT

Background: despite limited long-term survival, kidney transplantation is the best form of renal replacement therapy for terminal disease patients. Components of extracellular purinergic signaling plays a fundamental role on inflammation and immune response related to organ transplantation. They could be alternative targets to avoid graft rejection.
Materials and Methods: The hydrolysis of ATP, ADP and AMP nucleotides was analyzed in both

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lymphocytes and platelets, as well as the quantification of ATP and ADA activity. A sample of 30 patients who underwent kidney transplants was obtained, of which 15 had a transplant time of less than one year (acute response) and 15 had a transplant time between one and three years (chronic response).

Results: In the group with transplantation time between one and three years, it was possible to identify a significant decrease in the amount of ATP, increase in ATP hydrolysis in platelets, decrease in AMP hydrolysis and increase in ADA activity, also in platelets. In the lymphocyte sample, there was a significant reduction in ADA activity as well as a decrease in the amount of ATP.

Conclusions: From the data obtained in the study, it can be inferred that adenosine can reduce pro-inflammatory cytokines, providing greater graft survival and reducing the intensity of graft-versus-host disease. ATP signaling exerts inflammatory effects and modulates the purinergic signaling cascade, offering new avenues for drug therapies to combat chronic graft rejection.

Keywords: Kidney transplantation; purinergic molecules; adenosine triphosphate; chronic renal insufficiency.

1. INTRODUCTION

Renal function is gradually and progressively reduced in patients with chronic kidney disease (CKD), leading to a terminal condition of the disease. CKD can be developed by a series of pathological mechanisms in order to trigger damage to one or more renal compartments. Regardless of the factor that triggered the kidney injury, there is impairment of the tubular cells, so that these cells are replaced by collagen scars and densely infiltrated macrophages. These factors are associated with loss and functional failure of renal cells [1].

Kidney transplantation sustains life and is the best treatment for patients with terminal CDK, being the most efficient method of renal replacement therapy [2]. Immunosuppression, effectively over the years, is a necessity, however, unwanted side effects and toxicities are observed. To prevent allograft rejection, immunosuppressive therapies often target T lymphocytes or B lymphocytes, however, their efficacy is limited and with short-term survival [3]. Alternative approaches are needed to combat chronic graft rejection.

The molecules, receptors and ectoenzymes of the purinergic system could be an alternative measure regarding the process of combating graft rejection, since this system has important participants in metabolic processes, such as inflammatory and immunological responses [4-6]. Extracellular adenosine triphosphate (ATP) is either passively released by necrotic cells during cell damage, or it is actively released by activated immune cells and by apoptotic cells [7,8]. In addition, ATP binds to the purinergic receptors (P2 family) in immune cells, activating them. Thus, these cells can be released during the transplantation process, as well as after cell damage or cell death, in the peritransplantation period and during ischemia and reperfusion, acting as a danger signal and modulating the alloimmune response. Once present in the extracellular space, ATP can be hydrolyzed by ectonucleotidases, such as ectonucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1 or CD39) in adenosine diphosphate (ADP) and adenosine monophosphate (AMP), there are also involved in the immune modulation process [9]. AMP can be hydrolyzed by ecto-5'-nucleotidase (CD73), producing adenosine, which interact with purinergic receptors (P1 family) and usually, its responses are the opposite of that from P2 receptors stimulation [10]. Then, the enzyme adenosine deaminase (ADA) converts extracellular adenosine into inosine [11].

ATP is mainly associated with pro-inflammatory response; however, adenosine has opposite effects, limiting inflammation by suppressing the actions of immune cells [4,12-16]. Knowing that the purinergic signaling is involved in the inflammatory response that leads to acute kidney rejection and chronic allograft dysfunction, we evaluated the modulation of purinergic signaling components in patients after kidney transplantation.

2. MATERIALS AND METHODS

2.1 Chemicals and Equipment

The substrates adenosine 5′-triphosphate disodium salt, adenosine 5′diphosphate sodium

salt, 5′-monophosphate sodium salt, adenosine, bovine serum albumin, Trizma base, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Ficoll-Histopaque (Lymphoprep) and Coomassie Brilliant Blue G were obtained from Sigma-Aldrich (St. Louis, MO, USA). The centrifuge used was Sigma 3k-16® refrigerated and the rotors were changed depending on the samples.

2.2 Patients and Samples

The sample consisted of 15 kidney transplant patients with transplant time <1 year and 15 kidney transplant patients 1–3 years after transplantation. From all, a 10-mL of peripheral blood sample was obtained. It is an interventional research with a quantitative approach of descriptive and comparative character. The study was carried out at the Clínica Renal Oeste, Chapecó, Santa Catarina, Brazil, which serves patients with chronic kidney disease undergoing hemodialysis and patients who underwent kidney transplantation. Kidney transplant patients of both sexes, aged over 20 years and who are being followed up at the clinic, participated in the study. Patients over 75 years of age, patients who died and patients who lost the graft or dropped out of follow-up were excluded from the study.

2.3 Experimental Design

The selected patients have already been submitted to kidney transplantation, both women and men, and were being followed up at the health service. Participants over the age of 75 years were excluded, as well as those who died during the research time and individuals who lost the graft and/or gave up the survey. A form was used to obtain the clinical information regarding age, transplantation time, tobacco use and comorbidities. A blood sample was also collected to perform the procedures described below.

2.4 Platelets and Lymphocytes Separation

The methods of Pilla and col. [17] modified by Lunkes and col. [18] were used to prepare platelet-rich plasma. Total blood was collected with sodium citrate as anticoagulant and centrifuged at 1500 rpm for 10 minutes. Then, platelet-rich plasma was centrifuged at 5000 rpm for 30 minutes and washed with 3.5 mM HEPES buffer, pH 7.0 at least twice. The pellets of platelets were suspended in HEPES buffer and protein concentration was adjusted to 0.4–0.6 mg/mL.

The mononuclear leukocytes were isolated from human blood collected with Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA - K3) and separated on Ficoll-Histopaque density gradients as described by Boyum [19].

2.5 Protein Determination

The method of Bradford [20] was used to measure protein concentration, using bovine serum albumin as standard. This assay is based on the binding of the dye Coomassie Blue G-250 to protein, accompanied by measuring the maximum absorbance of the solution at 595 nm.

2.6 E-ntpdase and CD73 Assays

The method of Lunkes and col [18] was used to determine E-NTPDase activity, with the addition of ATP or ADP as substrate at a final concentration of 1.0 mM, to start the reaction. The method of Heymann and col [21] was used to determine CD73 activity. The hydrolysis of ATP, ADP and AMP releases phosphate, which was measured using potassium phosphate monobasic (KH_2PO_4) as standard. Controls were prepared to correct for nonenzymatic hydrolysis, and all samples were analyzed in triplicates. Enzyme activities are reported as nmol Pi released/min/mg of protein.

2.7 Quantitative ATP Determination

ATP was quantitatively determined using a commercial bioluminescence assay with recombinant firefly luciferase and its substrate D-luciferin. This assay is based on the ATP requirement of the luciferase enzyme to produce light, at a maximum emission of approximately 560 nm at pH 7.8.

2.8 Adenosine Deaminase (ADA) Determination

The method of Giusti and Galanti [22] was used to determine ADA activity on platelets and lymphocytes, based on the direct measurement of ammonia produced when ADA acts in an extracellular environment with excess of adenosine. Briefly, 50 µL of cells reacted with 21

mmol/L of adenosine, pH 6.5 was incubated at 37 °C for 60 minutes. Afterwards, the reaction was stopped by adding a solution of 106.2 mM phenol, 167.8 mM sodium nitroprussiate and a hypochlorite solution. The amount of ammonia produced was measures at 620 nm and the results were expressed in units per liter (U/l).

2.9 Statistical Analysis

Statistical analyses were performed using the software GraphPad Prism 7. The differences between the groups were evaluated through unpaired T test analysis, and the probability of rejection of the null hypothesis as being less than 5% ($p < 0.05$) were considered statistically significant.

3. RESULTS

3.1 Patient's Profile

We collected and summarized the clinical patients' characteristics (Table 1). The data were obtained through a documented interview with each participant. In relation to sex prevalence, we observed an increase in men (approximately 70%) than in woman (approximately 30%) in transplant patients. The average age was similar between groups (52 ± 2,49 and 46,2 ± 1,76 years old in <1 and 1-3 years after transplant, respectively; p= 0,068).

Considering the most common associated diseases in CKD patients, our studied group presented diabetes, hypertension and cardiac diseases. Diabetes prevalence was similar between groups (around 30%, p= 0,026), but hypertension was prevalent in patients with 1-3 years after transplant (80%, p= 0,008). Cardiac diseases were found more frequently in patients <1 year after transplant (13,3%), with no presence in patients 1-3 years after transplant.

3.2 Purinergic Signaling Parameters

To elucidate whether the ectoenzymes (with degrading purines) had altered activity, we evaluated the hydrolysis of the ATP (Fig. 1A) and ADP (Fig. 1B) nucleotides in lymphocytes, as well as the hydrolysis of ATP (Fig. 2A), ADP (Fig. 2B) and AMP (Fig. 2C) on platelets. The hydrolysis of AMP in lymphocytes is very small, so the technique employed was not sensitive enough for its analysis. The concentration of extracellular ATP showed a significant decrease at the group 1–3 years after transplant (Fig. 1D).

As can be seen, the hydrolysis of ATP (Fig. 1A) and ADP (Fig. 1B) in lymphocytes did not show any significant difference in the analyzed groups. Thus, E-NTPDase activity was not altered at different time points after transplantation. The same analyzes were performed on platelets, and we observed an increase in ATP hydrolysis in 1–3 years after transplant group (Fig. 2A). This result suggests an increase in E-NTPDase activity in platelets. ADP (Fig. 2B) and AMP (Fig. 2C) hydrolysis did not show significant differences between groups.

Furthermore, the activity of ADA on platelets (Fig. 2D) showed a significant increase in the group 1-3 years after transplant, differently from ADA activity in lymphocytes (Fig. 1C), in which there was a decrease in its action. Thus, our results indicated changes in some parameters of the purinergic cascade after a longer transplant period.

4. DISCUSSION

The best option of renal replacement therapy for patients with terminal CKD is the kidney transplantation, even if long term survival is limited due to the development of chronic allograft dysfunction, which develops a chronic inflammation process in the kidney, with interstitial fibrosis and tubular atrophy [3]. Extracellular ATP is released by leukocytes, endothelium, and platelets and provide environmental signals that could be involved in the inflammatory response that accompanies rejection and chronic allograft dysfunction [23]. Although the literature is poor in direct studies on the role of extracellular ATP in kidney transplantation, our results show for the first time, alterations on purinergic system parameters in patients with transplant time <1 year (acute response) and in patients 1–3 years after transplant (chronic response).

According to our findings, an increased release of ATP is likely to occur in acute phase (<1 year) of allograft transplantation, possibly due to ischemia, reperfusion or immune cells activation, acting as a danger signal that may modulate the alloimmune response [24]. In patients 1-3 years after transplant, we observed a decrease in ATP levels when compared to patients <1 year after transplant (Fig. 1D). This decrease may be

beneficial to patients and could favor no rejection with patients' long-term survival.

The ATP could induce inflammation in the organ after implantation and can mediate allograft rejection. Mice that received kidneys overexpressing human CD39 had fewer tubular injuries, better renal function and survived for longer than mice that received wild-type kidneys [11]. Other studies also suggest involvement of extracellular ATP in the regulation of cellular and immunologic process that occurs during allograft organ rejection. In heart, islet and lung transplantation models, extracellular ATP signaling has been associated with long-term graft disfunction [24], representing a possibly therapeutic target in kidney transplantation. E-NTPDase and CD73 ectoenzymes may represent future therapeutic targets as well. In a study with a mouse model of ischemia reperfusion injury (I/RI), an insult in transplantation related to periods of cold and warm ischemia during organ procurement, deletion of E-NTPDase was related to renal injury, however, administration of a soluble E-NTPDase improved renal function in wild-type mice [25].

Table 1. Clinical characteristics of kidney transplanted patients

	<1 year	1 – 3 years	p
Gender (%)			
Woman	26,6 (n= 4)	33,3 (n= 5)	0,026
Men	73,4 (n= 11)	66,7 (n= 10)	
Age (years)	52,00 ± 2,492	46,20 ± 1,763	0,068
Transplant time (months)	7,133 ± 0,9354	23,87 ± 1,884	< 0,0001*
Diabetes (%)	26,7 (n= 4)	33,3 (n= 5)	0,026
Hypertension (%)	33,3 (n= 5)	80 (n= 12)	0,008*
Cardiac disease (%)	13,3 (n= 2)	0	1,56 x10^{-6}*

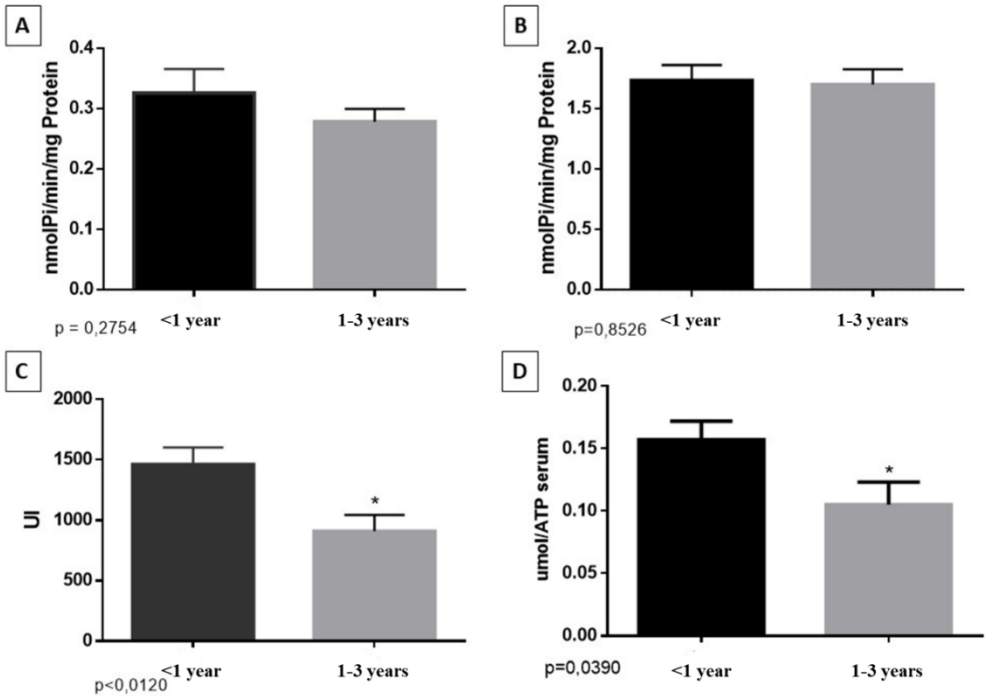

Fig. 1. ATP, ADP and adenosine hydrolysis in lymphocytes and quantitative ATP determination in serum of patients with transplant time <1 year, and 1–3 years after transplant

*Legend: A) ATP hydrolysis in lymphocytes by E-NTPDase; B) ADP hydrolysis in lymphocytes by E-NTPDase; C) Adenosine hydrolysis in lymphocytes by ADA; D) Quantitative serum ATP determination. Data are presented as means ± SD. Student's t test, n = 15, *p<0,05. ADA = adenosine deaminase, ATP = adenosine triphosphate, ADP = adenosine diphosphate, E-NTPDase = ectonucleoside triphosphate diphosphohydrolase, SD = standard deviation*

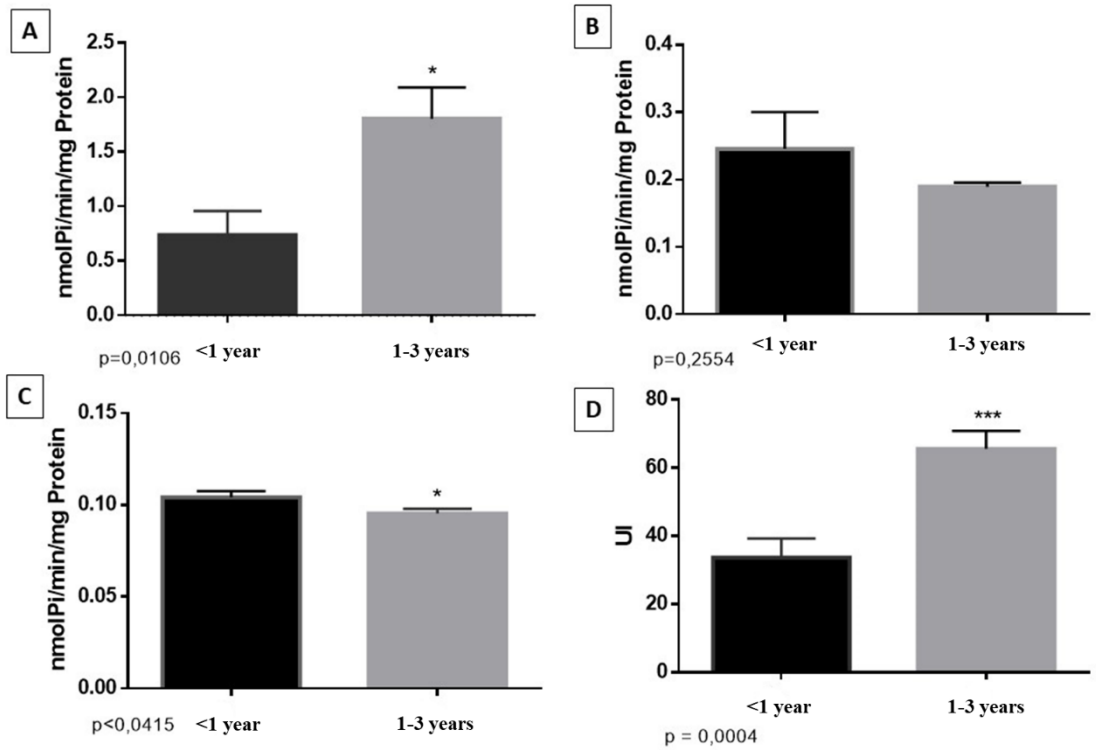

Fig. 2. ATP, ADP, AMP and adenosine hydrolysis in platelets of patients with transplant time <1 year, and 1–3 years after transplant

*Legend: A) ATP hydrolysis in platelets by E-NTPDase; B) ADP hydrolysis in platelets by E-NTPDase; C) AMP hydrolysis in platelets by CD73; D) Adenosine hydrolysis in platelets by ADA. Data are presented as means ± SD. Student's t test, n = 15, *p<0,05; ***p<0,0001. ADA = adenosine deaminase, ATP = adenosine triphosphate, ADP = adenosine diphosphate, AMP = adenosine monophosphate, E-NTPDase = ectonucleoside triphosphate diphosphohydrolase, CD73 = ecto-5'-nucleotidase, SD = standard deviation*

We evaluated E-NTPDase and CD73 activity in lymphocytes (Fig. 1) and platelets (Fig. 2) and observed an increase in E-NTPDase activity – ATP hydrolysis – in platelets on chronic group. Activated granulocytes release ATP to the extracellular environment, which accumulates at the vascular interface and promotes an increase in E-NTPDase activity. These ectonucleotidases are present at the endothelium membrane, and hydrolyses the extracellular ATP and ADP to AMP, which is then transformed by CD73 in adenosine. Adenosine inhibits platelet adhesion and aggregation. Increased ATP hydrolysis by E-NTPDase, but no ADP or AMP hydrolysis, suggests an accumulation of ADP, which binds to platelet P2Y12 receptor and promotes its activation [26].

Platelets play a crucial part in hemostatic and inflammatory processes and are associated with diverse inflammatory pathologies [27–29], which highlights the potential of targeting these cells to control the inflammatory and hemodynamic responses in transplanted patients [26,28]. It is interesting to note that in our study, in lymphocytes, although there is no change in E-NTPDase activity in acute or chronic transplanted patients, some studies show that genetic ablation of E-NTPDase and CD73 enhanced hypoxic injury in kidney and liver transplanted murine models and reduced cardiac allograft survival [3,23,30].

In addition to the above effects, the E-NTPDase and CD73 enzymes regulate extracellular adenosine signaling pathways. Loss of adenosine production increase the activity of T effector lymphocytes, typical in acute transplant rejection [11]. Our results suggest changes in adenosine metabolism, as we observed an increase in its hydrolysis in platelets (Fig. 1C) and a decrease in lymphocytes in patients 1-3 years after transplant.

Adenosine is a potent modulator of lymphocyte development, proliferation and activity, and its effect depends both on its bioavailability and on cell surface P1 receptors expression [31]. In

humans, the absence of ADA activity results in severe lymphopenia and immunodeficiency [32]. The observed decrease in ADA activity in these cells in our study, and the consequent accumulation of adenosine, may be related to graft rejection and resistance. While ATP emits a danger signal, adenosine is an immunosuppressive molecule, so its increase on chronic kidney transplanted patients could improve graft response and be a future therapeutic option.

The observed increase of ADA activity on platelets of chronic transplanted patients (Fig. 2D) could be explained by ADA removing of adenosine, which was produced by additional breakdown of ATP, ADP and AMP. Platelets are abundant in the peripheral blood and can develop a quick response to biological changes. The signaling pathways of the purinergic system are responsible for promoting immunosuppression and improving the inflammatory process. Adenosine can reduce the production of pro-inflammatory cytokines, thus, the graft resulting from kidney transplantation has longer survival, as well as reducing the intensity of the graft versus host disease. Furthermore, purinergic signaling is related to the regulation and direction of the inflammatory process due to local immune responses. Thus, the direction of purinergic signaling by increasing the activity of ectonucleotidases and/or by increasing immunosuppression via adenosine, can prevent vascular lesions of the allograft, thus providing less rejection and greater tolerance [26].

5. CONCLUSION

Based on the data obtained from this study, it was found that in patients with kidney transplantation time between 1-3 years, extracellular ATP levels were reduced. Thus, there may be a lower long-term rejection propensity, enabling graft survival. This is due to the fact that ATP is a pro-inflammatory molecule, and the reduction in the circulating levels of this molecule would reduce the activation of inflammatory mediators, so as to provide a favorable environment for the graft.

In summary, despite human study limitations, the whole of results suggests important data about the purinergic signaling in kidney transplant patients. Such understanding allows us to infer that ATP signaling exerts inflammatory effects, and may modulate the purinergic signaling cascade and, thereby, offers new avenues for drug therapies to combating chronic rejection to the graft.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

To participate in the study, all subjects gave written informed consent.

ETHICAL APPROVAL

The Human Ethics Committee of Federal University of Fronteira Sul, Chapecó, Santa Catarina, Brazil, approved the study under the protocol number 2.752.288.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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