RELATÓRIO TÉCNICO CIENTÍFICO FINAL

TÍTULO DO PROJETO: Transplante renal: Inter-relação dos sistemas da coagulação, fibrinolítico e inflamatório

CÓDIGO DO PROCESSO: CDS 535/04

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Julho/2008
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Benefícios obtidos:

- Maior conhecimento acerca da resposta imune humoral e mediada por células no transplante renal. Futuramente, tal conhecimento poderá reverter em medidas estratégicas de promoção à saúde de pacientes transplantados.

- Maior conhecimento do papel do óxido nítrico no processo de rejeição a órgãos transplantados.

- Maior conhecimento acerca das metodologias laboratoriais diretas e indiretas para determinação de óxido nítrico em amostras biológicas, o que poderá viabilizar a determinação deste radical em diversas situações clínicas.

- Consolidação da parceria entre a Faculdade de Farmácia-UFMG e o Centro de Pesquisa René Rachou-Fiocruz, com a criação de uma nova linha de investigação laboratorial: a determinação de óxido nítrico intracelular por citometria de fluxo. A determinação direta de óxido nítrico poderá constituir um método auxiliar para monitoração de outros estados inflamatórios e imunológicos, como os processos infecciosos e tumorais.

- Consolidação da parceria entre a Faculdade de Farmácia-UFMG e as Unidades de Transplante Renal do Hospital das Clínicas-UFMG e do Hospital São Francisco de Assis-BH, com possibilidade de desenvolvimento de outros estudos.

- Contribuição para a consolidação do Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia/UFMG.

Alcance dos resultados esperados:

- Maior conhecimento acerca dos aspectos imunológicos da rejeição renal, o que poderá reverter em medidas estratégicas de promoção à saúde de pacientes transplantados, refletindo na melhor evolução clínica e redução do tempo e número de internação.
- Inovação experimental com o estabelecimento do método de citometria de fluxo para determinação intracelular de óxido nítrico.

- Difusão do conhecimento por meio de artigos científicos a serem publicados e de resumos a serem apresentados em congressos.

- Formação de novas parcerias para trabalhos de pesquisa.

Considerações finais:

No projeto para o qual o auxílio financeiro foi concedido, intitulado “Transplante renal: Inter-relação dos sistemas da coagulação, fibrinolítico e inflamatório” (CDS 535/04), que seria a dissertação de Mestrado da aluna Natália Castro de Carvalho Schachnik, estava prevista a determinação de óxido nítrico em pacientes transplantados renais. Esse interesse decorreu de um estudo anterior (Dissertação de mestrado de Rívia Mara Morais e Silva, Programa de Pós-graduação em Ciências Farmacêuticas, sob nossa orientação, em 2004) onde o óxido nítrico foi avaliado indiretamente, pela determinação de nitrato e nitrito plasmáticos (Reação de Griess) e foi obtido um aumento desse radical em pacientes com rejeição renal aguda. Esse resultado motivou a investigação da produção de óxido nítrico utilizando um método direto e mais sensível, a citometria de fluxo. No entanto, o estabelecimento das condições laboratoriais para a determinação intracelular de óxido nítrico em monócitos e neutrófilos circulantes foi extremamente trabalhoso e demorado. Ficou patente o crescimento intelectual da mestranda Natália, responsável pelo trabalho, que teve de evoluir em vários aspectos para vencer todos os desafios laboratoriais. Dessa forma, optou-se pela conclusão do mestrado da Natália com o estudo intitulado “Estabelecimento do método de citometria de fluxo para determinação de óxido
nitríco em monócitos e neutrófilos circulantes e aplicação em pacientes com nefropatia crônica do enxerto".

O projeto original "Transplante renal: Inter-relação dos sistemas da coagulação, fibrinolítico e inflamatório" foi revisto e atualizado e constituirá o projeto de doutorado da aluna Ana Paula Lucas Mota. Parte do material de consumo para o desenvolvimento desse projeto já foi adquirida utilizando os recursos recebidos (Proc. CDS 535/04).

COMENTÁRIOS GERAIS E AGRADECIMENTOS

Como membro do Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia/UFMG, gostaria de agradecer o financiamento concedido, o que representa um estímulo à busca de novos conhecimentos e métodos laboratoriais e contribui para a consolidação de programas de Pós-Graduação.

Ressalto a importância destes financiamentos de pesquisa que possibilitam acompanharmos os avanços produzidos em outras partes do mundo e viabilizar o desenvolvimento e a soberania do país. Cumpre registrar também a grande contribuição ao ensino de Graduação, à medida que laboratórios são melhorados e professores se tornam cada vez mais preparados para exercer com mais competência e estímulo a difícil, mas gratificante, tarefa da transmissão do conhecimento.

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Luci Dusse

Profª Luci Maria Sant'Ana Dusse

Belo Horizonte, 29 de julho de 2009
UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMÁCIA

NATÁLIA CASTRO DE CARVALHO SCHACHNIK

ESTABELECIMENTO DO MÉTODO DE CITOMETRIA DE FLUXO PARA
DETERMINAÇÃO DE ÓXIDO NÍTRICO EM MONÓCITOS E
NEUTRÓFILOS CIRCULANTES E APLICAÇÃO EM PACIENTES COM
NEFROPATIA CRÔNICA DO ENXERTO

Belo Horizonte
2008
NATÁLIA CASTRO DE CARVALHO SCHACHNIK

ESTABELECIMENTO DO MÉTODO DE CITOMETRIA DE FLUXO PARA DETERMINAÇÃO DE ÓXIDO NÍTRICO EM MONÓCITOS E NEUTRÓFILOS CIRCULANTES E APLICAÇÃO EM PACIENTES COM NEFROPATIA CRÔNICA DO ENXERTO

Dissertação submetida ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia da Universidade Federal de Minas Gerais, como requisito parcial para obter o grau de mestre em Ciências Farmacêuticas.

Orientadora: Profª. Drª. Luci Maria Sant’Ana Dusse
Co-orientador: Dr. Olindo de Assis Martins-Filho
Co-orientadora: Drª. Vanessa Peruhype-Magalhães

Belo Horizonte
2008
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Linha de Pesquisa:
Desenvolvimento e Aplicações Analíticas em Química Biológica e Toxicologia

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40101053- Hematologia

Instituições participantes:
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Hospital das Clínicas / UFMG
AGRADECIMENTOS

A Deus, pela vida e porque sem Ele nada seria possível.

À professora Luci Maria Sant'Ana Dusse pela amizade, confiança e incentivo desde a graduação e pela orientação e dedicação neste trabalho.

Ao Olindo de Assis Martins Filho, pelos ensinamentos sobre citometria de fluxo e pela enorme contribuição neste trabalho.

À Vanessa Peru hype Magalhães, pela amizade, ensinamentos e pelas longas horas de trabalho e dedicação em várias etapas deste estudo.

Aos professores do Departamento de Análises Clínicas e Toxicológicas da Faculdade de Farmácia, em especial professora Maria das Graças Carvalho e professor Lauro Mello Vieira, por estarem sempre dispostos a me ajudar, principalmente durante o pós-doutorado da professora Luci.

À farmacêutica Geralda de Fátima, pelo auxílio em diversas etapas deste trabalho. Ao Jarbas, pelas longas e divertidas conversas.

A FAPEMIG, CNPq e CAPES, pelo apoio financeiro.
Dear Luci Dusse,

Your submission entitled "Use of flow cytometry for the analysis of nitric oxide in monocytes and neutrophils of the peripheral blood of patients with chronic graft nephropathy" has been received by Journal of Immunological Methods.

You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/jim/.

Your username is: Luci Dusse
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Thank you for submitting your work to this journal.

Kind regards,

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Journal of Immunological Methods
Use of flow cytometry for the analysis of nitric oxide in monocytes and neutrophils of the peripheral blood of patients with chronic graft nephropathy

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Abstract

Nitric oxide (NO) is one of the most important biosynthesized molecules and participates as a mediator in various biological processes. Increasing evidence suggests a role for the NO resulting from inducible NO synthase (iNOS) as a mediator in the graft rejection process. The laboratorial determination of NO represents a challenge principally because of its unreliable half-life and concentration in biological samples. Recently, flow cytometry was proposed as a method for the determination of intracellular NO with 4,5-
diaminofluorescein diacetate (DAF-2DA). In the present study, the protocol for the determination of intracellular NO in monocytes and neutrophils of peripheral blood by flow cytometry was optimized, and the profile for the production of this compound in individuals with chronic graft nephropathy (N=11) and healthy individuals (N=10) was determined. No difference in the production of NO was observed in the two groups; although a lower impact of LPS on the percentage of DAF-2T⁺ monocytes of patients with chronic graft nephropathy was observed comparing with the healthy control group. This minor impact of LPS was not observed in neutrophils, suggesting that the use of immunosuppressive drugs reduce the response of monocytes, but not of neutrophils, to stimulation with LPS. It is possible that the production of intracellular NO in monocytes and neutrophils of patients with chronic graft nephropathy is suppressed by immunosuppressive drugs. However, the application of flow cytometry for the determination of NO in other processes in which the participation of monocytes and neutrophils is important and NO has cytotoxic and cytostatic action (inflammatory/infectious and tumorous processes) would certainly be of great value.

Keywords: Nitric oxide, chronic graft nephropathy, flow cytometry, DAF-2DA.

1. Introduction

The chronic nephropathy of kidney grafts stems from continuous immune and non-immune aggression of the transplanted kidney. It is characterized by progressive, irreversible, functional and morphological deterioration of the renal graft and may occur months or years after the transplant. This slow and variable loss of renal function is frequently linked to proteinuria and hypertension (Paul, 1999; Krieger et al. 2003). Increasing evidence suggests a role for nitric oxide (NO) resulting from inducible NO-synthase (iNOS) as a mediator in the process of graft rejection. NO is the principal cytotoxic mediator of activated immune effectors cells and constitutes an important
regulator molecule of the immune system (Tripathi, 2007). Increased synthesis of NO has been reported in kidney rejection, both in animal models and in humans (Lu et al., 1999; Albrecht et al., 2000). The direct determination of NO in biological samples is a challenge primarily because of its miniscule half-life and concentration (Kiechle and Malinski, 1993).

Recently, flow cytometry was proposed as a method for determining intracellular NO using 4,5-diaminofluoresceína diacetate (DAF-2DA). This method is based on the ability of DAF-2DA to penetrate the cell cytoplasm, where it is converted into 4,5-diaminofluoresceína (DAF-2), and on the oxidation of NO, which leads to the formation of triazolofluoresceína (DAF-2T). This triazolic product (DAF-2T) emits a green fluorescence, whose intensity can be measured in a flow cytometer and is proportional to the concentration of intracellular NO (Kojima et al., 1998). This study sought to examine the production of intracellular NO in monocytes and neutrophils of the peripheral blood of patients with chronic nephropathy of the grafted kidney using flow cytometry.

2. Material and methods

Ethics approval was granted for the study by the Research Ethics Committee of the Federal University of Minas Gerais. Samples were only collected after each individual received detailed information about the study and had given informed consent.

2.1. Population

Patients who had undergone kidney transplantation at the São Francisco de Assis Hospital, Belo Horizonte, Brazil and the Clinical Hospital, Federal University of Minas Gerais, Brazil, and who had progressed to chronic graft nephropathy (N=11) were included in this study, as well as clinically healthy individuals who constituted the healthy control group (N=10). The post-transplant period ranged from 1 to 96 months, and all the patients used immunosuppressants, especially cyclosporin, tacrolimus, and mophenyli
mycophenolate associated with glucocorticoid (prednisone). Patients with infectious processes detected by a leukogram and by a routine examination of the urine, as well as those recently vaccinated, were excluded.

2.2 Sample

A 10mL sample of venous blood was collected in sodium heparin from all the participants in the study using the Vacutainer (Becton-Dickinson-Mountain View, CA, USA) system of tubes and needles. The samples were processed 24 hours after collection.

2.3 Reagents

Buffered saline solution, pH 7.2; FACS lysing solution - Becton-Dickinson-Mountain View, CA, USA; human monoclonal antibody anti-CD14 TC and anti-CD16 TC - Caltag; 4,5-diaminofluorescein diacetate (DAF-2DA) - Calbiochem; lipopolysaccharide from Escherichia coli (LPS) - Sigma; aminoguanidine (AG) - Sigma, N-nitro-L-arginine methyl ester (L-NAME) - Sigma.

2.4 Method

The establishment of the flow cytometry method to determine the intracellular NO in monocytes and neutrophils in the peripheral blood was based on the original protocols described by HAVENGA et al. (2001) and STRIJDOM et al. (2004) for analysis of NO in vascular cells and cardiomyocytes, respectively. Three experimental conditions were evaluated: basal production of NO, NO production in the presence of the AG inhibitor, and production of NO in the presence of the LPS inducer. All three conditions were evaluated in duplicate to allow for the subsequent labeling with anti-CD14 TC and anti-CD16 TC antibodies in separate tubes.

To determine the baseline production of NO, 50µL of plasma-free whole blood was
incubated for 180 minutes in an oven at 37°C in the presence of DAF-2DA (final concentration=2 μM) and 5% CO₂. To determine the production of NO after inhibition with AG, 50 μL of plasma-free whole blood was incubated for 10 minutes in the presence of AG, at a of 10 mM, followed by incubation in an oven at 37°C for 180 minutes in the presence of DAF-2DA (final concentration=2 μM) and 5% CO₂.

To determine the production of NO after stimulation with LPS, 50 μL of plasma-free whole blood was incubated for 60 minutes in the presence of LPS (final concentration=10 μg/mL), followed by incubation in an oven at 37°C for 180 minutes in the presence of DAF-2DA (final concentration=2 μM) and 5% CO₂.

After incubation for 180 minutes in the presence of DAF-2DA, the tubes were transferred to an ice bath and incubated for 20 minutes in the presence of 0.5 μL of anti-CD14 TC or anti-CD16 TC. Stained samples were treated by gently stirring on a vortex stirrer with 2 mL of FACS Lysing Solution (Becton Dickinson Biosciences Pharmigen, San Diego, CA) and re-incubated for an additional 10 minutes at room temperature. After erythrocyte lysis was complete, the white blood cells were washed twice with 1 mL of phosphate-buffered saline and centrifuged at 1300 rpm for seven minutes at 4°C and 18°C, respectively, the supernatant was discarded and the cell pellet was gently re-suspended. Finally, 200 μL of phosphate-buffered saline was added, and the contents were transferred to 5 mL polystyrene tubes for immediate reading in the flow cytometer. A total of 50,000 events/tube were obtained using the FACScan ® (BD) flow cytometer. The CELLQuest software was used for the acquisition and analysis of data.

3. Statistics

Data are presented as medians, and statistical analysis was performed with the GraphPad Prism 4.00 (San Diego, CA). Comparison of medians was accomplished using ANOVA, Kruskal-Wallis and Dunn’s method or the Mann-Whitney method. Values of P<0.05 were
considered as significant.

4. Results

4.1 Establishment of the flow cytometry method for analysis of intracellular NO in monocytes and neutrophils in peripheral blood

4.1.1 Pre-treatment of whole blood samples

Two protocols for pre-treatment of whole blood samples were evaluated: isolation of the buffy coat and the use of plasma-free whole blood. The analysis of morphometric and immunophenotypical profiles from isolated white blood cells showed that the use of plasma-free whole blood promoted a better segregation of the monocyte populations with a phenotype characteristic of CD14$^{\text{High}^+}$ cells, based on the immunophenotype profile for labeling with anti-CD14 TC. Moreover, this protocol caused less distortion of the morphometric monocyte profile, as demonstrated by the more homogeneous and compact distribution relative to that obtained by the isolation of the buffy coat, as is presented in Figure 1.

4.1.2 Determination of the optimum DAF-2DA concentration

Plasma-free total blood samples were incubated in the presence of DAF-2DA, at final concentrations of 0.2µM, 2.0µM and 4.0µM, to determine the optimum DAF-2DA concentration. The percentage of DAF-2T$^+$ cells and the immunophenotype profiles obtained with the use of DAF-2DA in different concentrations were taken into consideration in choosing the optimum DAF-2DA concentration. The 2.0µM concentration of DAF-2DA presented the highest percentage of DAF-2T$^+$ the cells without any distortion of the immunophenotype profile of the white blood cells, as is presented in Figure 2A.

4.3 Profile of intracellular NO monocytes and neutrophils in the two groups
The production of NO by monocytes (A) and neutrophils (B) of the peripheral blood of patients with chronic graft nephropathy and healthy control individuals, and the impact of stimulation with LPS and inhibition with AG are shown in Figure 5.

The data show distinct profiles in the analysis of intracellular NO monocytes and neutrophils in the peripheral blood of patients with chronic graft nephropathy when compared to the control group of healthy individuals. These differences were more apparent in the analyses of the differential impacts of LPS and the AG on monocytes and neutrophils. Additional analysis of the results were made to compare the profiles of DAF-2T+ cells in the population of monocytes and neutrophils, as well as the impact of LPS and the AG on these two cell populations (Figure 6).

4.1.3 Defining the ideal incubation time for the samples with DAF-2DA
Plasma-free total blood samples were incubated for 60, 120, 180 and 240 minutes in the presence of a 2.0μM concentration of DAF-2DA for the definition of the ideal incubation period for the samples with DAF-2DA. The results of the incubation kinetics are represented in Figure 2B. There was a progressive increase in the percentage of DAF-2T+ monocytes as a function of the period of incubation with DAF-2DA. However, the magnitude of the increase was higher in the intervals between 120 to 180 minutes (2.0 times) than in the other intervals evaluated (60 to 120 minutes, 1.1 times; 180 to 240 minutes, 1.4 times). Thus, the protocol employing the 180-minute incubation period was selected.

4.1.4 Selection of the best inducer and inhibitor of NO production
For the selection of the best inducer for the production of NO, samples of plasma-free whole blood were pre-incubated for 60 minutes in the presence of different inducing agents for NO production, including phorbol 12-myristate 13-acetate (PMA) at a final
concentration of 0.25μg/mL, PMA plus ionomycin at a final concentration of 0.25μg/mL and 1.0μg/mL, concavaline A (ConA) at a final concentration of 64μg/mL, phytohemagglutinin (PHA) at a final concentration of 100μg/mL and lipopolysaccharide from *Escherichia coli* (LPS) at a final concentration of 10μg/mL, followed by incubation for 180 minutes in the presence of DAF-2DA (final concentration= 2.0μM). The percentage of DAF-2T⁺ cells and the immunophenotypic profiles obtained with the use of different inducers were considered in the selection of the best inducer of NO production in intracellular monocytes. The use of LPS resulted in the largest increase in the percentage of DAF-2T⁺ cells without the occurrence of any distortion in the immunophenotype profile of white blood cells, as is shown in Figure 3A. For selecting the best inhibitor of intracellular NO production in monocytes, samples of plasma-free whole blood were pre-incubated for 10 minutes in the presence of two inhibitors of NO production, N⁵-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AG), followed by incubation for 180 minutes in the presence of DAF-2DA (final concentration=2.0μM). The results for the impacts of different inhibitors on the percentage of DAF-2T⁺ monocytes are presented in Figure 3B. Data analysis revealed a greater impact of inhibition on the percentage of DAF-2T⁺ monocytes in the protocol that employed AG.

4.2 Acquisition and analysis of data for evaluating the profile of intracellular NO production in circulating monocytes and neutrophils by flow cytometry

The different strategies used to analyze the NO profile in intracellular subpopulations of leukocytes are represented in Figure 4. The selective analysis of monocytes was established by the combination of anti-receptor markers of the cell surface (FL-3) versus laser side-scatter (SSC) to discriminate and select the monocytes as SSC⁺CD14⁺ cells (Figure 4A). Following the selection of the monocyte population, the percentage of DAF-2T⁺ monocytes was determined using quadrant statistics of FL-1/DAF-2TFITC versus
FL-3/anti-CD14 TC dot plots (Figure 4B).

The selection of the neutrophil population was performed using the graph of FL-3/anti-CD16 TC versus laser side-scatter (SSC), and the neutrophils were discriminated as SSC$^{\text{High}}$CD16$^{\text{High}}$ cells (Figure 4C). Following the selection of the neutrophil population, the percentage of DAF-2T$^+$ neutrophils was determined using quadrant statistics of FL-1/DAF-2TFITC versus FL-3/anti-CD16 TC dot plots (Figure 4D).

All results were expressed as the percentage of DAF-2T$^+$ cells, corresponding to the upper right quadrant - double positive (Q2).

4.3 Profile of intracellular NO in monocytes and neutrophils in the two groups

The production of NO by monocytes (A) and neutrophils (B) of the peripheral blood of patients with chronic graft nephropathy and healthy control individuals, as well as the impact of stimulation with LPS and inhibition with AG are presented in Figure 5. The data show distinct profiles in the analysis of intracellular NO in monocytes and neutrophils of patients with chronic graft nephropathy when compared to the control group. These differences were more apparent in the analyses of the differential impacts of LPS and AG on monocytes and neutrophils. Additional analysis of the results were performed to compare the profiles of DAF-2T$^+$ cells in the population of monocytes and neutrophils, as well as the impact of LPS and the AG on these two cell populations (Figure 6).

5 Discussion

A role for NO in the process of rejection of a transplanted organ has been demonstrated. Ouyang et al. (2005) showed that the use of a specific inhibitor of iNOS (FR260330) was able to prevent the occurrence of chronic rejection of the aorta in rats, thereby demonstrating the role of NO from iNOS in this type of rejection. Vos et al. (2004)
suggested that NO derived from iNOS is related to the occurrence of the rejection of grafts, while the NO derived from cNOS appears to protect the transplanted organ. Albrecht et al. (2002) showed that, just as in acute rejection, an increase in the expression of iNOS in the interstice and a reduction in glomerular expression of eNOS, associated with increased ROS formation, occurs during the chronic failure of the transplanted kidney. Reports in the literature suggest that one of the mechanisms of action of immunosuppressant drugs is the inhibition of iNOS, with the consequent decrease in production of NO. Kaibori et al. (1999) showed that tacrolimus inhibits the expression of mRNA for iNOS in a rat hepatocyte culture, when these cells were stimulated with IL-1β. However, this effect was not observed with the use of cyclosporin.

In contrast to this finding, Attur et al. (2000) demonstrated that cyclosporin and rapamycin inhibited the expression of iNOS mRNA in a macrophage culture after immune stimulation with LPS. This inhibition was not observed with the use of tacrolimus. Lessio et al. (2005) also showed that cyclosporin inhibited the expression of iNOS in renal artery cells from rats, suggesting that the reduction of NO could be related to cyclosporin nephrotoxicity.

Tuñón et al. (2003) have shown that tacrolimus and rapamycin inhibited the production of NO and the expression of mRNA for iNOS in an in vitro culture of LPS-stimulated hepatocytes from rats. It is known that mycophenolate mofetil is able to inhibit the in vitro synthesis of NO. Lui et al. (2001), in an experiment with mice, found that the mycophenolate mofetil was able to reduce the in vivo expression of mRNA for iNOS and the production of NO in the kidney under ischemic conditions.

This study did not show an increase in the production of NO by circulating monocytes and neutrophils in patients with chronic graft nephropathy comparing to healthy controls. One justification for this result is the use of immunosuppressive drugs by all patients with chronic graft nephropathy, since these drugs are able to inhibit the synthesis of NO,
primarily by inhibiting iNOS.

Another relevant datum in this study was the lower impact of LPS on the percentage of DAF-2T+ monocytes of patients with chronic graft nephropathy than on the healthy control group (Figure 6B). This minor impact of LPS was not observed in neutrophils comparing the two groups. This fact suggests that the use of immunosuppressive drugs reduce the response of monocytes, but not of neutrophils, to stimulation with LPS. One possible explanation for this phenomenon could be the lower constitutive expression of CD14 by the population of neutrophils relative to the population of monocytes. It is known that the induction of NO production by LPS is mediated by the interaction of LPS with the CD14/TLR-4 complex (Gangloff et al., 2005). This could justify the lower degree of induction of NO production mediated by LPS in the population of neutrophils and, consequently, the differentiated profile of the impact of LPS on the NO production in monocytes and neutrophils. The results in Figure 6B corroborate this hypothesis, showing that, in the presence of LPS, a greater impact of the percentage of DAF-2T+ cells on the population of monocytes than on that of the neutrophils occurs in the control group.

Regarding the impact of AG on the percentage of DAF-2T+ cells, the data suggest that, in the microenvironment of immunosuppression, the neutrophils become resistant to inhibition by AG (Figure 6C). In an attempt to explain this phenomenon, it was hypothesized that individuals submitted to immunosuppression could display distinct profiles of NOS isoforms in neutrophils and monocytes. Another option to be investigated would be that neutrophils of patients with chronic graft nephropathy would exhibit a distinct profile of NOS isoforms, with a lower contribution of iNOS in neutrophils than in monocytes. The results in Figure 6C corroborate this hypothesis, showing that there is a lower impact on the percentage of DAF-2T+ cells in the population of neutrophils than on the monocytes of patients with chronic graft nephropathy in the presence of AG, a selective iNOS inhibitor. Further studies will be needed to investigate this hypothesis.
Another observation that is relevant for the analysis of the results obtained in this study is the use of glucocorticoids by patients with chronic graft nephropathy. The glucocorticoids, such as prednisone and prednisolone, are regulators of gene transcription and are present in most immunosuppressive schemes. The glucocorticoids inhibit the proliferation of T cells, the T cell-dependent immunity and the expression of genes that encode cytokines (IL-1, IL-2, IL-6, α-TNF, γ-IFN). Moreover, they have anti-inflammatory activity, being able to inhibit the iNOS (Thompson, 2003). This inhibition could explain why an increase in the production of NO by monocytes and neutrophils in the peripheral blood was not observed despite the fact that the chronic graft nephropathy is a process associated with inflammation.

In conclusion, the analysis of the profile of intracellular NO production in circulating monocytes and neutrophils of patients with chronic graft nephropathy was accomplished by flow cytometry. In pathological processes, in which the participation of monocytes and neutrophils is important and the NO possesses cytotoxic and cytostatic action (inflammatory / infectious and tumorous processes), the application of flow cytometry as an auxiliary method will certainly be of great value.

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References


Figure 1 - Morphometric and immunophenotypical profiles of monocytes after in vitro culture employing leucocitary cream or plasma-free whole blood. Less distortion of the immunophenotype profile characteristic of monocytes, such as CD14^{High} cells, was observed in the protocol employing plasma-free whole blood.

Figure 2 - (A) Morphometric and immunophenotypical analysis of DAF-2T^{+} monocytes after in vitro culture of plasma-free whole blood in the presence of different concentrations of DAF-2DA. The results are expressed as an average percentage±standard deviation of DAF-2T^{+} monocytes. Graphs representative of a point distribution by density illustrate the morphometric and immunophenotypical profiles of monocytes obtained in each protocol. (B) Percent of DAF-2T^{+} monocytes after various periods of in vitro culture employing samples of plasma-free whole blood in the presence of 2.0μM DAF-2DA. The results are expressed as a percentage of DAF-2T^{+} monocytes in each protocol assessed. Analysis of changes in the percentage of DAF-2T^{+} monocytes assessed at different times (Δ = T120/T60; T180/T120 and T240/T180) are represented in the graph.

Figure 3 - (A) The impact of pre-incubation of samples of plasma-free whole blood for 60 minutes with different inducers of NO production, followed by incubation for 180 minutes in the presence of 2.0μM DAF-2DA, on the morphometric and immunophenotypical profile of monocytes. The results are expressed in the form of the impact of different NO inducers on the percentage of DAF-2T^{+} monocytes [((Inducer/DAF-2DA x 100) - 100]. Representative graphs of the point distribution by density illustrate the morphometric and immunophenotypical profiles of monocytes obtained in each protocol. (B) The impact of pre-incubation with different inhibitors of NO production for 10 minutes on the percentage of DAF-2T^{+} monocytes after incubation of samples of plasma-free whole blood for 180 minutes in the presence of 2.0μM DAF-2DA. The results are expressed in the form of the
impact of different inhibitors of NO production on the percentage of DAF-2T\(^+\) monocytes 
\[100 - (\text{Inhibitor/DAF-2DA} \times 100)\].

Figure 4 - Strategies used to analyze the profile of intracellular NO in circulating monocytes and neutrophils.

Figure 5 - Production of intracellular NO by monocytes (A) and neutrophils (B) of the peripheral blood of patients with chronic graft nephropathy and healthy control individuals. The results are expressed as median (horizontal bar) and dispersion of individual values (symbols) of the percentage of DAF-2T\(^+\) monocytes observed in cultures performed in the presence of only DAF-2DA (\(\bigcirc\)) as well as cultures submitted to stimulation with LPS (\(\blacktriangle\)) and inhibition with AG (\(\bigtriangleup\)). The statistically significant differences (P<0.05) are represented by the letters "a" and "c" for cultures performed only in the presence of DAF-2DA and cultures subject to inhibition with AG, respectively.

Figure 6 - Production of intracellular NO by monocytes and neutrophils of the peripheral blood of healthy control subjects and patients with chronic graft nephropathy. The results are expressed as medians (horizontal bar) and maximum and minimum percentages of DAF-2T\(^+\) cells observed in cultures performed in the presence of only DAF-2DA (A), after stimulation with LPS (B), and inhibition with AG (C). Statistically significant differences (P<0.05) in the production of NO in monocytes and neutrophils are represented by \(*\).
Figure 3

A

Impact on DAF-2T⁺ monocytes %

PMA  PMA+IONO  Con-A  PHA  LPS

B

Impact on DAF-2T⁺ monocytes %

L-NAME  AG
Dear Colleague,

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DIRECT NITRIC OXIDE DETECTION IN MONOCYTES AND NEUTROPHILS BY FLOW CYTOMETRY IN KIDNEY TRANSPLANTED PATIENTS
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Nitric oxide (NO) is one of the most important biosynthesized molecules and participates as a mediator in various biological processes. The laboratorial determination of NO represents a challenge principally because of its unreliable half-life and concentration in biological samples. Recently, flow cytometry was proposed as a method for the determination of intracellular NO with 4,5-diaminofluorescein diacetate (DAF-2DA). Increasing evidence suggests a role for the NO resulting from inducible NO synthase (iNOS) as a mediator in the graft rejection process. Renal transplant is the treatment of choice for patients that are in the final stage of kidney disease and the chronic nephropathy of the graft is the most important cause of loss of a transplanted kidney. In the present study, the protocol for the determination of direct intracellular NO in circulating monocytes and neutrophils by flow cytometry was optimized, and the profile for the production of this compound in kidney-transplanted individuals with graft rejection process (n = 11) and healthy individuals (n = 10) was determined. No difference in the production of NO was observed in the two groups. The production of NO in circulating monocytes and neutrophils in patients with graft rejection process is probably suppressed as a result of the action of immunosuppressors.

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Otimização da metodologia para determinação de óxido nítrico intracelular por citometria de fluxo

Relatório Final Atividades
Pró-Reitoria de Pesquisa
Programa Institucional de Iniciação Científica

Felipe Carlos Brito de Souza
2004020827
Relatório de Atividades Final

PARTE 1

1.1. Programa: PIBIC/CNPq  
1.2. Modalidade de relatório: Final  
1.3. Identificação:  
   Nome do bolsista: Felipe Carlos Brito de Souza  
   Número de Matrícula: 200402827  
   Nome do curso: Farmácia  
   Nome do orientador: Luci Maria Sant’Ana Dusse  
   Projeto de pesquisa: Inter-relação dos processos da coagulação, fibrinolítico e inflamatório em pacientes transplantados renais  
   Projeto avaliado por: COEP (Cópia da aprovação, em anexo)  
   Número do protocolo de entrega: ETIC 415/05  
   Unidade/Departamento: Faculdade de Farmácia/ Departamento de Análises Clínicas e Toxicológicas/ Laboratório de Hematologia Clínica

1.4. Data de ingresso como bolsista no Programa em vigência: Agosto de 2005

1.5. Indique: Bolsa Nova


1.7. Resumo do plano de trabalho do aluno apresentado no início da bolsa  
   - Extensa revisão bibliográfica.  
   - Colaboração na elaboração de projeto de pesquisa.  
   - Colaboração no preenchimento do formulário próprio para envio do projeto à COEP/UFGMG e na elaboração da ficha clínica.  
   -Participação na otimização da metodologia para análise de óxido nítrico intracelular.

1.8. Relatório de atividades do bolsista.

1.8.1 Atividades realizadas:  
   1) Revisão bibliográfica do assunto sobre citometria de fluxo e transplante renal a partir de artigos científicos, dissertações e materiais especializados diversos;  
   2) Participação na otimização da metodologia para determinação de óxido nítrico intracelular.