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Efeito imunomodulador de hipoglicemiantes orais em cultura de linfócitos de pacientes com diabetes tipo 2

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key words	abstract
<p>Diabetes mellitus</p> <p>Chlorpropamide</p> <p>Metformin</p> <p>Immunomodulation</p>	<p>Introduction and objective: It has been suggested that type 2 diabetes is an inflammatory response manifestation. The main drugs used to treat type 2 diabetes are sulphonylureas and biguanides. The aim of this study was to demonstrate the modulatory effects of oral hypoglycemic drugs (chlorpropamide and metformin) on lymphocyte proliferation <i>in vitro</i> and <i>ex vivo</i>. Methods: Peripheral blood mononuclear cells were isolated from human blood by gradient centrifugation. T-lymphocytes were stimulated by phytohemagglutinin (PHA) and oral hypoglycemic drugs. Results: In both <i>in vitro</i> and <i>ex vivo</i> experiments, there was a reduction in cell proliferation after treatment with oral hypoglycemic drugs. When both drugs were used in combination, a high level of cytotoxicity was observed, which made analysis of immunomodulatory effects unfeasible. Discussion and conclusion: We demonstrated that diabetes itself may reduce cell proliferation significantly when stimulated by PHA, which may indicate that diabetic patients have difficulties in promoting an efficient inflammatory response. Moreover, the use of oral hypoglycemic drugs may aggravate this situation.</p>

resumo	unitermos
<p>Introdução e objetivos: Tem sido sugerido que o diabetes mellitus tipo 2 (DM2) é uma manifestação da resposta inflamatória. As principais drogas utilizadas no tratamento do DM2 são as sulfonilureias e as biguanidas. O objetivo deste trabalho é demonstrar os efeitos moduladores na proliferação de linfócitos causada pelos hipoglicemiantes orais (clorpropamida e metformina), <i>in vitro</i> e <i>ex vivo</i>. Métodos: Células mononucleares de sangue periférico foram isoladas de seres humanos por gradiente de centrifugação. Os linfócitos T foram estimulados com fito-hemaglutinina (PHA) e hipoglicemiantes. Resultados: Nos experimentos <i>in vitro</i> e <i>ex vivo</i>, mostramos a redução da proliferação celular quando do tratamento com drogas hipoglicemiantes orais. Quando as drogas foram utilizadas em combinação, foi observado alto grau de citotoxicidade, tornando inviável a análise do efeito imunomodulador. Discussão e conclusão: Mostramos que o diabetes, por si, pode reduzir significativamente a proliferação celular quando estimulada por PHA, o que pode indicar que o paciente diabético tem dificuldade em promover a eficiente resposta inflamatória e que o uso de hipoglicemiantes pode piorar esta situação.</p>	<p>Diabetes mellitus</p> <p>Clorpropamida</p> <p>Metformina</p> <p>Imunomodulação</p>

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Introduction

Diabetes *mellitus* is a metabolic disorder characterized by a congenital or acquired inability to transport sugar from the bloodstream into cells. The underlying problem in diabetes mellitus is usually either an inadequate secretion of insulin or a reduction in the tissue responsiveness to insulin. Type 2 diabetes is the most prevalent form of the disease, although it is frequently asymptomatic in initial periods, leading to the possibility of remaining without a diagnosis for many years⁽¹⁾. Indeed, its prevalence has increased globally, including many parts of the developing world, as a consequence of the worldwide obesity epidemic⁽¹⁴⁾.

It has also been suggested that type 2 diabetes is an inflammatory host response manifestation⁽²²⁾. Several studies have shown that inflammation markers, such as C-reactive protein, fibrinogen, and pro-inflammatory cytokines, for instance interleukin-6 and tumor necrosis factor- α (TNF- α), are associated with type 2 diabetes^(6-8, 21). Moreover, some authors have shown that decreased interleukin-10 production capacity is associated with type 2 diabetes⁽²⁵⁾. There is also evidence that the insulin resistance seen in sepsis is proportional to the inflammation severity⁽¹³⁾.

Sulphonylureas and biguanides are the main drugs used in the treatment of type 2 diabetes. Sulphonylureas stimulates, in a direct effect, insulin secretion by binding to a receptor in the pancreatic beta-cell plasma membrane, resulting in ATP-sensitive K⁺ channels inhibition, membrane depolarization and calcium influx through voltage-dependent Ca²⁺ channels. The sulphonylureas action upon K⁺ channels can present crossed reactivity with cellular receptors in other tissues (heart, skeletal and smooth muscles) than the pancreas^(2, 19). Representing the first generation of sulphonylureas, the chlorpropamide has a powerful hypoglycemic activity and a long time effect. Because of that, they can induce a great number of hypoglycemic episodes. Thus, it is contraindicated in elderly and nephropatic patients⁽²⁾. Previous study has shown that an immunomodulatory effect of chlorpropamide *in vitro* is possible. Reduction in the culture cell proliferation was observed when stimulated by LPS (lipopolysaccharide of *Escherichia coli*), as well as some pro-inflammatory cytokines expression reduction, such as the soluble TNF- receptor type II⁽¹⁷⁾.

On the other hand, metformin, a type of biguanides, doesn't have effects on insulin absence or deficiency. Metformin reduces the hyperglycemia by increasing peripheral sensitivity to insulin, catalyzing the tyrosine-kinase

activity and reducing the glucose production through gluconeogenesis, as well as limiting the intestine glucose absorption. The drug can either be used alone or associated to the sulphonylureas^(2, 12).

Thus, the aim of the present work was to demonstrate the possible oral antidiabetic drugs (sulphonylureas and biguanides) modulatory effects on the lymphocytes proliferation evaluated *in vitro* and *ex vivo*.

Materials and methods

Subjects

Healthy volunteers and diabetic type 2 patients were selected according to the inclusion and exclusion criteria. Blood samples were collected and a pharmacovigilance questionnaire was performed. The study was submitted and approved by the Pontifícia Universidade Católica do Rio Grande do Sul Ethical Committee (150/04). All samples from patients and volunteers were used only in the present study analysis and were kept until its conclusion and/or rejected as a request from a participant.

Criteria of inclusion

1. Patients and volunteers that, after being fully informed about the study, gave written consent accepting to participate. The consent was obtained prior to the blood collection, after the consent letter was carefully read by the subjects in the presence of a researcher, that was available to give any additional information;
2. to answer a "Pharmacovigilance Questionnaire", that was presented to the patients previously to the blood collection and right after the written consent was given.

Criteria of exclusion

1. Primary or secondary immunosuppressive diseases;
2. corticosteroids therapy use or any other type of drug with immunosuppressive effects;
3. decompensate type 2 diabetes (characterized for values of glycated hemoglobin > 8%).

Experiment 1

Blood samples from healthy volunteers were collected and the peripheral blood mononuclear cells separated. The following groups were performed according to the treatment used: chlorpropamide ($n = 5$), metformin ($n = 5$) and chlorpropamide + metformin ($n = 5$). We have

evaluated both lymphoproliferation and cytotoxicity. In the lymphoproliferation assay a group of unstimulated cells and mitogen (phytohemagglutinin) stimulated cells were used as controls. On the other hand, in the cytotoxicity assay only a control group of isolated cells were used.

Experiment 2

Subjects were divided in five groups according to the pharmacovigilance questionnaire answers: control group (non-diabetic subjects – $n = 5$), diabetic patients treated with controlled diet ($n = 5$), diabetic patients treated with chlorpropamide ($n = 5$), diabetic patients treated with metformin ($n = 5$) and diabetic patients treated with combined therapy (chlorpropamide + metformin) ($n = 5$). Lymphoproliferation was evaluated.

Preparation of peripheral blood mononuclear cells (PBMCs)

The PBMCs were isolated through gradient centrifugation on Ficoll-Paque (Sigma) and resuspended in RPMI 1640 (Invitrogen) supplemented with 0.15% garamicin (Schering-Plough) and 20% homologous serum at a final cell density of $1.6 \times 10^6/\text{ml}$ ⁽¹⁸⁾. Platelet contamination of these preparations was less than 1%. The viability was measured by trypan blue dye exclusion and was uniformly greater than or equal to 90%⁽²⁰⁾.

Lymphoproliferation assay

Phytohemagglutinin (PHA) (Invitrogen) was used for T-lymphocyte proliferation. Chlorpropamide and metformin were dissolved in RPMI 1640. PBMCs (1.6×10^5 cell/well) were plated in a 96-well microtiter bottomed flat plates (Nunc) and incubated at 37°C in a 5% CO₂-humidified incubator for 96 hours. PHA (10 µg/ml) was added to induce proliferation of the T-lymphocytes. Chlorpropamide (5 mM) and metformin (1 mM) were also added to the cultures (*in vitro*), according to the experimental groups. Lymphocyte proliferation was determined by MTT (3-[4-5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Invitrogen) assay⁽¹⁵⁾. Briefly, MTT was dissolved in RPMI 1640 at 5 mg/ml and added to all wells of the assay. Plates were then incubated at 37°C for four hours. Isopropanol (Quimex) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes, the plates were read on a Hyperion MicroReader,

using a test wavelength of 540nm and a reference wavelength of 650 nm. The experiments were performed on different days and each experiment was done in triplicate.

Cytotoxicity assay

Chlorpropamide, metformin and the combined therapy were dissolved in RPMI 1640 and added directly to the PBMCs (1.6×10^5 cell/well), which were incubated in a 96-well microtiter bottomed flat plates at 37°C in a 5% CO₂ humidified incubator. The cellular viability was performed by trypan blue dye exclusion after 96 hours of the incubation^(17,18,20). The experiments were performed on different days and each experiment was done in triplicate. The results are presented as percentage.

Statistical analysis

Statistical analyses were performed using SPSS version 16.0 for Windows. Data were analyzed for significance using one-way analysis of variance (ANOVA). The Bonferroni's *post-hoc* test was used for the multiple group comparisons and significance was established when $p < 0.05$. Data are presented as mean \pm standard error of the mean (SEM) and all measures were performed in triplicate.

Results

The immunomodulatory effect of sulphonylurea and biguanide isolated or combined on *in vitro* lymphoproliferation in cultures using healthy volunteers cells stimulated with PHA was evaluated. Results presented in **Figure 1** show that all treatments significantly decreased proliferation of these cells. Moreover, the cells that received the combined drug treatment also presented a reduction, although it was much more pronounced. In order to determine whether the inhibitory effect of chlorpropamide and metformin on lymphoproliferation was due to cellular death, the cellular viability was investigated. As shown in **Figure 2**, in the cells treated with isolated drugs, no alterations were seen. However, when both drugs were combined the cell viability was significantly reduced.

After that, we have performed an experiment to evaluate the effects of these drugs, isolated or combined, when used in a therapeutic dosage. Lymphocytes from type 2 diabetic patients were used in this case. **Table** presents the characterization of the patients, including age, time of treatment and glycated hemoglobin (HbA1c), to evaluate

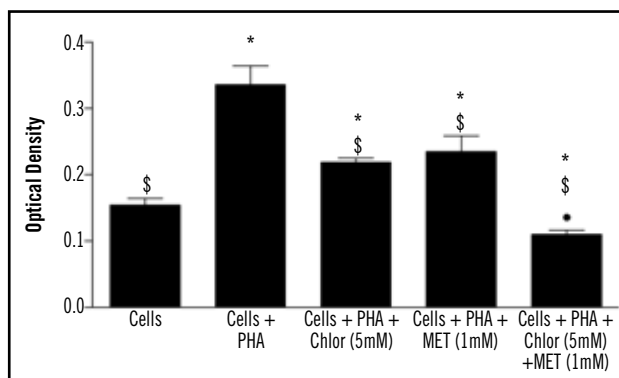


Figure 1 – In vitro lymphoproliferation assay using healthy subjects' cells treated with oral antidiabetic drugs. *Indicates significant differences ($p < 0.05$) when non-stimulated cells (Cells) were compared to the other groups. \$ Indicates significant differences ($p < 0.05$) when stimulated cells (Cells + PHA) were compared to the other groups. • Indicates a significant difference ($p < 0.05$) when compared to the other treatment groups

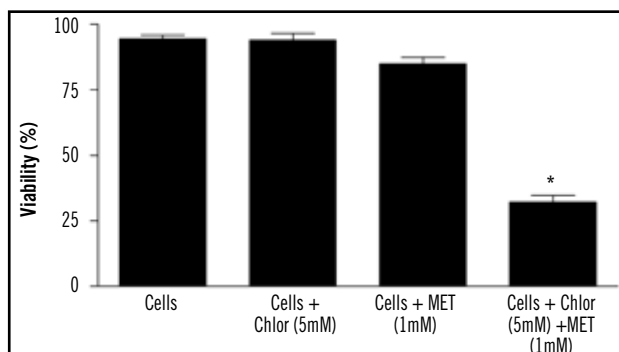


Figure 2 – In vitro cytotoxicity assay using healthy subjects' cells treated with oral antidiabetic drugs. *Indicates a significant difference ($p < 0.05$) when the combined therapy group was compared to the control group (Cells)

Characterization of the diabetic patients: age, time of treatment and glycated hemoglobin

Table

Treatment	Age (years)	Time of treatment (months)	Glycated hemoglobin (%)
Diet	53 ± 5	43 ± 53	6.3 ± 0.8
Chlopropamide	62 ± 20	88 ± 128*	6.4 ± 1.4
Metformin	56 ± 10	38 ± 82	5.7 ± 0.9
Chlopropamide + metformin	59 ± 10	67 ± 53	6 ± 2

Significant difference between chlopropamide and the other groups are indicated by * ($p < 0.05$).

the control of diabetes. No significant differences were found when age and HbA1c were compared between groups. However, patients who had received sulphonylureas presented a significant higher ($p < 0.05$) time of treatment when compared to the other groups.

In order to assess the lymphoproliferation capacity of type 2 diabetic patients cells, an *ex vivo* assay was performed. As presented in **Figure 3**, diabetic patients' cells had significantly decreased proliferation when compared to non-diabetic subjects. Besides, it was shown that cells from patients who received treatment that included the concomitant use of chlorpropamide and metformin presented a significant reduction in the proliferation when compared to the other groups.

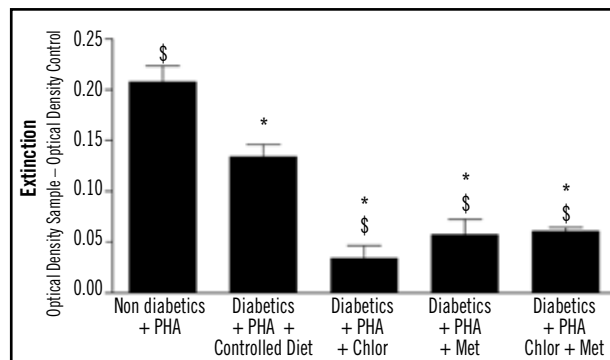


Figure 3 – Ex vivo lymphoproliferation assay using diabetic type 2 patients' cells that had used oral antidiabetic drugs or controlled diet in their treatment. *Indicates significant differences ($p < 0.05$) when all groups were compared to the non-diabetic group. \$ Indicates significant differences ($p < 0.05$) when all groups were compared to the diabetic patients with controlled diet cells group.

Discussion

Present study shows that all treatments used (chlorpropamide, metformin or both) induced an immunosuppression *in vitro*, although when drugs were tested in combination a cytotoxicity effect was demonstrated. The same immunosuppressive effect was also present in the *ex vivo* experiments, since both diabetic pharmacologically untreated and treated patients also showed a lymphoproliferation decrease.

Type 2 diabetes *mellitus* – a worldwide pandemic health problem⁽⁴⁾ – is a progressive disorder and, although oral antidiabetic drug monotherapy is often initially successful, it is associated with a high secondary failure rate, which contributes to the development of long-term diabetes complications resulting from persistent hyperglycemia. For patients not taking insulin, accumulating evidence suggests that combination therapy using oral antidiabetic agents with different mechanisms of action may be highly effective in achieving and maintaining target blood glucose levels. In the course of the disease, the use of combinations of oral agents may delay the need for insulin while maintaining glycemic control, thus making aggressive oral treatment more acceptable for many patients⁽³⁾.

The sulphonylureas, a class of drugs used in type 2 diabetes treatment, acts stimulating the insulin secretion in the pancreatic cells through the blockage of ATP-sensitive K⁺ channels. Experiments that relate structure and function of the ATP-sensitive K⁺ channels have provided important explanations in the mechanisms that support the sulphonylureas selectivity and the potential consequences of the blockage of these channels in pancreatic cells. ATP-sensitive K⁺ channels in pancreatic cells present different properties in comparison with cardiac and muscular tissues. They are stimulated by the expression of alternative types of sulphonylureas receptors with not-identical binding sites. The sulphonylureas present two main types of receptors, SUR1 in pancreatic cells e SUR2 in extra pancreatic tissues, with similar action potentials^(9, 10). The ATP-sensitive K⁺ channels structure have two extensions in helix (45Kda) that presents one binding site to the ATP. This channel locus is very similar to the voltage-dependent K⁺ channels basic structure, which could indicate that this channel region can share a common origin with the voltage-dependent K⁺ channel⁽¹¹⁾. The structural similarity between the ATP-sensitive K⁺ channels (susceptible to the sulphonylureas action) and the voltage-dependent K⁺ channels can help in the binding efficiency of the sulphonylureas in the cells, although it can lead to side effects in extra pancreatic tissues. The voltage-dependent K⁺ channels have a crucial role in the T-lymphocyte human activation, controlling the membrane rest potential⁽²³⁾. K⁺ channel blockers inhibit IL-2 production and lectin-induced T-lymphocyte proliferation⁽¹⁸⁾. The specific K⁺ channel blockers reversibly inhibit proliferation of human T-cells, without displaying cytotoxicity or blocking Ca²⁺-independent T-cell stimulatory pathways.

Metformin, a biguanide derivative, decreases hepatic glucose production through adenosine monophosphate-activated protein kinase activation⁽⁵⁾. Metformin does not activate 5'-AMP-activated protein kinase (AMPK) directly, but rather complex I in the respiratory chain, an effect likely to explain the life-threatening side effect of lactic acidosis⁽¹⁶⁾. AMPK is a multisubunit enzyme that is recognized as a major regulator of lipid biosynthetic pathways due to its role in the phosphorylation and inactivation of key enzymes, such as acetyl-CoA carboxylase. More recent data strongly suggest that AMPK has a wider role in metabolic regulation: this includes fatty acid oxidation, muscle glucose uptake, expression of cAMP-stimulated gluconeogenic genes and glucose-stimulated genes associated with hepatic lipogenesis, including fatty acid synthase. Chronic activation of AMPK may also induce the expression of

muscle hexokinase and glucose transporters (Glut4), mimicking the effects of extensive exercise training⁽²⁶⁾. Recently studies have shown that AMPK is dramatically but transiently activated when quiescent T-cells are stimulated via the antigen receptor. Quiescent T-cells have a very limited energy turnover but when stimulated, they need to generate a considerable amount of ATP for the rapid growth and proliferation that follows. That activation of AMPK allows them to rapidly switch on catabolic, ATP-generating processes to anticipate this demand. However, there is now much evidence that a high AMPK activity also represses cell growth and proliferation; therefore, it may be necessary for AMPK activity to return to baseline values before T-cell proliferation can commence⁽²⁴⁾.

In the *in vitro* experiments, we have shown a cell proliferation reduction after treatment with the oral antidiabetic drugs at specific concentrations. Considering the chlorpropamide results, we believe that this action could be associated to the blockage of the ATP-sensitive K⁺ channels, since K⁺ channels blockers have immunosuppressant activity⁽²³⁾. On the other hand, when the metformin results are analyzed, it's possible that this action could be associated to a stimulation of AMP-activated protein kinase. When both drugs were used in combination, a high level of cytotoxicity was demonstrated, making the immunomodulatory effect impossible to analyze.

In the *ex vivo* experiment, we have demonstrated that the diabetes, by itself, decreases lymphoproliferation. The mechanisms involved are not yet very clear. On the other hand, the cells of the patients treated with sulphonylureas, metformin or both, have showed the same results seen in the *in vitro* experiments, for instance a proliferation inhibition. Although the precise plasma concentration of each drug couldn't be determined, in all cases the therapeutic concentrations were effective in maintaining diabetes under control.

In the present study, we have demonstrated that diabetes itself can significantly reduce the cell proliferation when stimulated by PHA, which could indicate that diabetic patients have difficulties to execute an efficient inflammatory response and the use of oral antidiabetic drugs can worsen this situation. Thus, considering the immunomodulatory effects of these drugs, they could also increase the patients' susceptibility to infections, leading to an inefficient immunological response.

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