Research Article

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Effect of Ace Inhibitor in Pancreatic Islets of Diabetic and Hypercholesterolemic Rabbits, in *in Vivo* and *in Vitro* Study

Daniel Roberto Pomaro[1], Silvia Saiuli Miki Ihara[1], Aparecida Emico Hirata[2], Tatiana Helfenstein[3], Maria Cristina Izar[3], Tania Leme da Rocha Martinez[4*], Anita L R Saldanha[4], Lech Michal Szymanski[4] and Francisco Antonio Helfenstein Fonseca[3]

[1]*Department of Pathology, Universidade Federal de São Paulo, São Paulo, Brazil*

[2]*Department of Physiology, Universidade Federal de São Paulo, São Paulo, Brazil*

[3]*Department of Medicine, Universidade Federal de São Paulo, São Paulo, Brazil*

[4]*Nephrology Depatment, BP - A Beneficência Portuguesa de São Paulo, São Paulo, Brazil*

***Correspondence author**

Tania Leme da Rocha Martinez
BP - A Beneficência Portuguesa de São Paulo
Rua Comandante Ismael Guilherme,
358 - Jardim Lusitânia 04031-120 - São Paulo
SP, Brazil

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Abstract

Background: *Diabetes Mellitus is a major risk factor for vascular disease and the imbalance of the renin-angiotensin system (RAS) exerts deleterious effects on various organs, and so, the use of angiotensin-converting enzyme (ACE) inhibitor or angiotensin II type 1 receptor (AT1) blockers may have a beneficial effect. The activation of RAS in the pancreas islet may induce the synthesis of reactive oxygen species, causing oxidative stress-induced B cell dysfunction.*

Objective: *The effect of the ACE inhibitor was analyzed in pancreas of diabetic and hypercholesterolemic rabbits in in vivo model, and in isolated rabbit pancreatic islets incubated with high glucose medium in in vitro study.*

Methods: *We performed a study on tissue samples of pancreas of alloxan-induced diabetic rabbits which have also become hypercolesterolemic through a rich cholesterol chow. The animals were divided into four groups according to glucose levels (Group I=432±45 mg/dL, Group II=514±40 mg/dL, Group III=149±09 mg/dL, Group IV=156±10 mg/dL). The rabbits in groups II and IV received quinapril (30 mg/day) in their food and all animals received a diet enriched with 0.5% cholesterol for 12 weeks. The histopathology and histomorfometry of pancreas islets were realized in specimens stained by hematoxilin-eosin and immunohistochemical reaction with insulin antibody. In in vitro study, the production of superoxide was determined by hidroetidina oxidation and the fluorescence stimulated and quantified by confocal microscope.*

Results: *In in vivo study, destruction of beta cells was observed in diabetic animals, however, differences weren't observed in groups treated with ACE inhibitor analyzed by immunohistochemistry. In in vitro study, reduced production of superoxide was observed in the pancreatic islets treated with IECA in medium with low glucose concentration.*

Conclusion: *The ACE inhibitor impaired the production of superoxide in islets isolated from rabbits, mainly in the presence of normal concentration of glucose, suggesting that the blockade of RAS may attenuate the deleterious actions of Angiotensin II on pancreatic islet structure.*

Abbreviation

ACE : Angiotensin-Converting Enzyme
ANG II : Angiotensin II
AT1 : Angiotensin II type 1 receptor
HEt : Hydroethidine
RAS : Renin-Angiotensin System
ROS : Reactive Oxygen Species
SEM : Scanning Electron Microscopy

Introduction

Diabetes Mellitus is a major risk factor for vascular disease and the imbalance of the Renin-Angiotensin System (RAS) exerts deleterious effects on various organs. Activation of the RAS is associated with "classic" risk factors for coronary artery disease, such as diabetes (Hollenberg et al, 2004), hypertension (Volpe et al, 2002) and hypercholesterolemia (Singh & mehta, 2001), and the RAS is recognized as a

complex system capable of accelerating the development of atherosclerosis. Significant reduction in cardiovascular risk in diabetic individuals treated with an angiotensin-converting enzyme (ACE) inhibitor has been observed, and this reduction is independent of blood pressure (Yusuf et al, 2000). Thus, studies with ACE inhibitors demonstrate that the use of this drug is efficient, not only in normalizing blood pressure but, mainly, in controlling cardiovascular risks. Protection by ACE inhibitors use in atherosclerosis has been experimentally observed even without changes in blood pressure (Hoshida et al, 1997).

The RAS plays an important role in the endocrine pancreas, and recent studies suggest that its blockade can prevent new cases of diabetes by greater anatomical and functional preservation of the islets of Langerhans (Tyrberg et al, 2001; Aizman et al, 2010). Chronic exposure to angiotensin II (Ang II) increases oxidative stress (De Ciuceis et al, 2005; Piro et al, 2002) activates fibrogenesis (Kim et al, 2008), and promotes apoptosis (Aizman et al, 2010), which would imply the progressive loss of β-cell function, mainly seen in type 2 diabetes. Several factors seem to influence β-cell loss, including functional overload as a result of chronic hyperglycemia, presence of free fatty acids and oxidative stress (Piro et al, 2002). Although Ang II is known for its significant effects on cell proliferation and apoptosis, the contribution of RAS to the dynamic regulation of islet structure and function has not been well elucidated.

Several experimental studies with hypercholesterolemic rabbits have shown that the use of ACE inhibitors decreases the development of atherosclerotic lesions (Hoshida et al, 1997; Pomaro et al, 2005; Fonseca et al, 2003; Pomaro et al, 2006), being that the proposed mechanism for the benefit of ACE inhibitors in experimental atherosclerosis would be the action on cytokines that mediate the inflammatory response (Misiakos et al, 2001).

Alloxan-treated rabbits are used to study diabetes related disorders and the effects of hyperglycemia on the development of atherosclerosis. Alloxan administration in these animals leads to a deficit in insulin production similar to type 1 diabetes mellitus developed in humans (Miller et al, 1984) Morphological studies show that in the induction of diabetes by alloxan, the β cells of the pancreas are destroyed which results, after a period of about five days, in severe and persistent reduction in serum insulin, reduction in the number of β cells and weight loss body. In β cells, alloxan damages DNA, mitochondria, lysosomes and plasma membrane and leads to an imbalance in Ca^{2+} metabolism, but the reason for selective toxicity of alloxan to pancreatic β cells remains unknown (Tyrberg et al, 2001). In an experimental study, (Tikellis et al, 2004) demonstrated that RAS is upregulated in pancreatic islets of ZDF rats (Zucker Diabetic Fatty) and is associated with disruption of islet architecture, fibrosis, and apoptosis. This is consistent with observations of the RAS in other tissues, in which locally released Ang II plays an important role in regulating both organ structure and function. In the experimental model used, the RAS blockage significantly attenuated islet damage and increased β-cell mass, possibly due to a reduction in oxidative stress, decrease in apoptosis and attenuation of fibrosis. Chu et al, (2006) confirmed a greater activation of the RAS in db/db mice, and correlated this activation with insulin secretion, showing that the increase in Ang II occurred concomitantly with the lower insulin expression in an inversely proportional way. Interestingly, pretreatment of these animals with an Ang II type I receptor blocker was associated with a better functional response of pancreatic β cells to hyperglycemic stimulation. Thus, ACE inhibitors as described above have demonstrated multifunctional benefits far beyond their hemodynamic properties, especially on oxidative stress and on the inflammatory mechanism.

Objective

The activation of RAS in the pancreas islet may induce the synthesis of reactive oxygen species, causing oxidative stress-induced B cell dysfunction. The effect of the ACE inhibitor was analyzed in pancreas of hypercholesterolemic and alloxan-treated rabbits and in the isolated rabbit pancreatic islets incubated with high glucose medium.

Methods

Animals and experimental design

The protocol was approved by the Ethics in Research Committee of the Federal University of São Paulo. We performed a retrospective study on tissue samples of pancreas of alloxan-induced diabetic rabbits which have also become hypercholesterolemic through a rich cholesterol fed. Diabetes was induced in New Zealand white male rabbits, by a single dose of alloxan monohydrate (100mg i.v., Sigma) and the blood glucose level was determined after one week. The animals were allocated into four groups according to the glucose levels and treatment: I (n=10) and II (n=8) had glucose levels ≥250 mg/dL whereas III (n=12) and IV (n=12) had glucose level lower than 250 mg/dL. The animal in the groups II and IV were treated with an ACEI, quinapril (Accupril® Pfizer) (30mg/d) added to 100 g of chow. All animals were fed a 0.5% cholesterol-rich diet for 12 weeks and at the end of the experiment, the glucose levels were checked again to confirm formation of groups. Daily consumption was individually and carefully monitored throughout the study. After 12 weeks the animals were sacrificed with an overdose of xylazine (Rumpun, Bayer) and ketamine (Ketalar, Parke-Davis), and fragments of pancreas were collected for histopathological examinations. it was analyzed the histopathologic and the histomorphometric changes in samples of pancreas and an in vitro assay using isolated islets from rabbits was performed to evaluate the oxidative stress

Histology: Histological fragments of pancreas were analyzed on HE-stained slides, considering the following parameters: destruction of beta cells in pancreatic islets, presence of vacuolization in the pancreatic parenchyma and the ratio of number of nuclei per islet area. The islet area was measured from images captured with the aid of a Sony CCD-IR15 video camera (Sony, Japan), using a publicly available morphometric software (Image Tool for Windows vs. 3.0) from the Science

Center website of Health at the University of Texas.

Immunohistochemistry: Immunohistochemical analysis was performed on the histological sections of the pancreas using the anti-insulin antibody (1:200 Santa Cruz, USA). The immunohistochemical assay used was the indirect immunoenzymatic method in three stages, using the avidin-biotin-peroxidase strep to complex (LSAB – DAKO corp. USA). Development was performed with substrate H_2O_2 + chromogen 3.3 diaminobenzidine (DAB) (Sigma, Germany).

Histomorphometric analysis of the pancreas: 10 images were captured at 200x magnification (of each slide/animal) containing a marked islet in the pancreatic tissue submitted to immunohistochemical assay with anti-insulin antibody with a video- Sony CCD-IR15 camera (Sony, Japan) connected to an Olympus BX-40 microscope. Images were digitized, transmitted to a microcomputer and treated using the Corel Photopaint X4 program, Corel Corp. USA.

The photomicrographs of the islets (Figure 1A and 1B) were treated to evidence, through the creation of "masks", and it was possible to determine the area of each islet in addition to the area stained by the anti-insulin antibody (Figure 1C and 1D) using a software of publicly available morphometry (Image Tool for Windows vs. 3.0) from the University of Texas Health Science Center website to measure areas (μm^2).

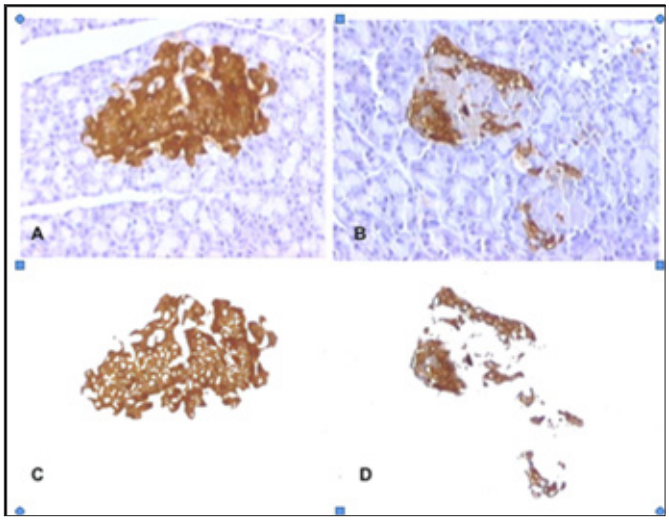

Figure 1: Pancreatic islets labeled with anti-insulin antibody. Image treatment for quantification by histomorphometry. (400x).

Evaluation of superoxide production in isolated rabbits pancreatic islets

The production of intracellular superoxide was determined by the oxidation of hydroethidine (HEt) (Molecular Probes, Eugene, USA). Rabbit pancreatic islets were collected in a petri dish with the aid of a magnifying glass after digestion with collagenase (Sigma-Aldrich, St Louis USA) and transferred to 24-well culture dishes.

The islets were then incubated for 60 minutes at 37°C in Krebs-Henseleit/KH Buffer in the presence of 22.2 mM glucose (Group I), 22.2 mM glucose and 1 mM ACE inhibitor (quinapril) (Group II), 5.5 mM glucose (Group III) and 5.5 mM glucose and 1 mM ACE inhibitor (Group IV).

Then, 100 µM of HEt was added to each well and the islets were incubated for another 20 minutes at room temperature. The incubation medium containing HEt was removed and the islets were washed with Krebs-Henseleit medium. Islets were visualized under a confocal microscope (Carl-Zeiss, Morris Plains, NJ, USA) and the fluorescent product of oxidation was excited to 547 nM and 3 µm confocal laser scanning slices were analyzed.

Statistical analysis:Data are expressed as mean±SEM. Statistical analysis was performed using the 1-way ANOVA test followed by the Newman-Keuls test if differences were observed. When necessary, the non-parametric Kruskal-Wallis test was performed. The unpaired t test (parametric data) was used to compare two independent groups. Categorical data were compared by the χ^2 test. Statistical significance was accepted with a p value <0.05. All analyzes were performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA).

Results

Histopathological changes in the pancreatic islets of rabbits that received treatment with alloxan were observed, with destruction of beta cells in the pancreatic islets in the diabetic groups. Vacuolization was observed in the pancreatic parenchyma of diabetic animals, resulting from the present hyperglycemia (70% and 62.5% of animals in groups I and II, respectively) (Table 1). The number of nuclei per islet area did not show significant difference, due to the smaller islet size in diabetic animals.

Group	n	Islet alteration (%)*	Vacuolization (%)*	Nucleous/ 100µm²
I	10	9 (90%)	7 (70%)	0,46 ± 0,08
II	8	5 (62,5%)	5 (62%)	0,42 ± 0,04
III	11	0 (0%)	0 (0%)	0,60 ± 0,05
IV	11	1 (9,1%)	1 (9,1%)	0,61 ± 0,07

Values expressed as mean ± standard error.
*p<0.0001; Fisher's exact test.

Table 1: Histological alterations in pancreas of diabetic and hypercholesterolemic rabbits treated or not with angiotensin-converting enzyme inhibitor.

Destruction of insulin-producing cells were observed, whose pancreatic islets showed lesser staining with anti-insulin antibody in the immunohistochemical reaction in the diabetic groups (Figure 2). Treatment with ACE inhibitors showed no significant change in relation to the untreated group, both in diabetics and non-diabetics (Table 2).

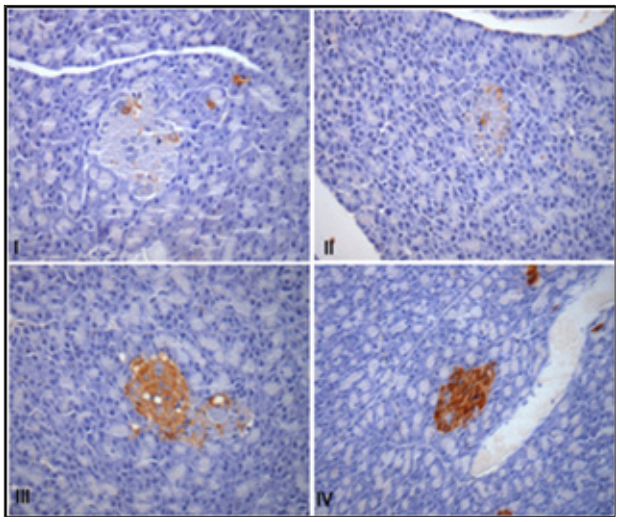

Figure 2: Photomicrograph of rabbit pancreas; Anti-insulin Ac immunohistochemistry (400X). Groups I and II < III and IV ($p<0.05$)

Group	n	Islet área (um^2)*	Positive area (μm^2)	Positivity %*
I	10	9973±777*	2317±775	21,44±5,54*
II	8	9194±883*	2052±589	22,74±5,31*
III	11	13150±935	3900±697	30,27±4,67
IV	11	15200±1726	4826±767	37,35±3,60

*p<0,05 I e II < III e IV - ANOVA and Newman Keuls Test

Table 2: Immunohistochemical staining with anti-insulin antibody in pancreatic islets of hypercholesterolemic and hyperglycemic rabbits treated or not with angiotensin-converting enzyme inhibitor.

***In vitro* oxidative stress test in pancreatic islets:** Oxidative stress was measured in an in vitro test with isolated rabbit pancreatic islets and subjected to different concentrations of glucose (22.2 mM - Group I and II and 5.5 mM Group III and IV) under treatment or not of the inhibitor of ACE (Groups II and IV treated with 1 mM of ACEi), mimicking the groups of animals and 57, thus evaluating the in situ production of superoxide anions by the fluorescent dye HEt in a confocal microscope (Figure 3). In this in vitro study, we observed less oxidative stress in isolated pancreatic islets treated with ACE inhibitors (Table 3).

Group	Mean (Pixel)	Standard deviation
I	21,50	11,23
II	13,56	7,78
III	16,50	8,70
IV	1,30*	1,30

* p<0,05 – IV < I e III, ANOVA and Newman Keuls Test.

Table 3: In situ production of superoxide anions in isolated rabbit pancreatic islets evidenced by the fluorescent dye hydroethidine.

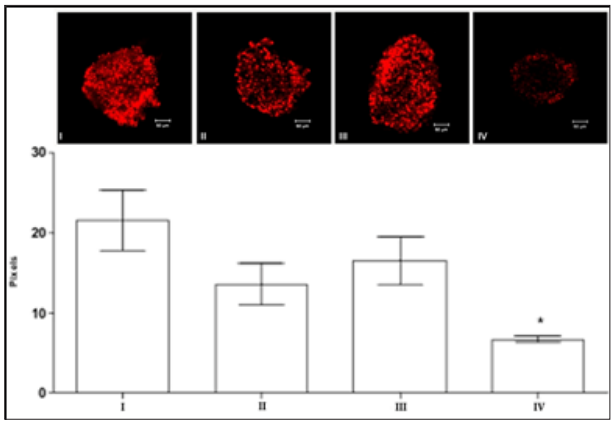

Figure 3: Confocal image of pancreatic islets marked by the conversion of hydroethidine to ethidium. (A) rabbit pancreatic islet incubated in medium with 5.5 mM glucose without treatment. (B) rabbit pancreatic islet incubated in medium with 5.5 mM glucose treated with 1 mM angiotensin-converting enzyme inhibitor.

Discussion

In *in vivo* study, destruction of beta cells in diabetic animals was observed, however, differences weren't observed in groups treated with ACE Inhibitors analyzed by immunohistochemistry. In the study with isolated rabbit pancreatic islets, we observed that the production of ROS by incubated islets determined by the HEt assay was significantly lower in islets treated with ACE inhibitors and incubated with 5.5 mM glucose, and this protection against oxidative stress was not observed in islets treated with ACE inhibitors and incubated with 22.2 mM glucose.

Similar to what it was observed in the many organs, the increase in glucose concentration abolished the benefits of using ACE inhibitors. These data suggest that one of the mechanisms by which diabetes could abolish the benefits of the use of ACE inhibitors would be via oxidative stress. In the pancreas, the protection exerted by RAS inhibitors on beta cell function is quite complex. Postulated mechanisms include increased potassium levels with improved insulin secretion by beta cells (Ferrannini et al, 1994), increased blood flow and perfusion of pancreatic islets, and anti-fibrotic and anti-apoptotic effect in islets (Tikellis et al, 2004). In addition, RAS inhibitors prevent beta-cell glucotoxicity (Lupi et al, 2006), primarily by reducing oxidative stress. These effects could slow or reverse the decline in beta cell function in predisposed individuals. Furthermore, the pancreatic hypersecretion of Ang II impairs the insulin signaling pathway in several cells, whereas RAS inhibitors could improve them (Henriksen et al, 1999).

In type 2 diabetes, alterations in post-insulin receptor signaling have been demonstrated (Lupi et al, 2006), such as abnormalities in phosphatidyl inositol-3 kinase and protein kinase B (Carvalho et al, 2016), and the suppression in the action of these enzymes can be accentuated 65 by Ang II and, therefore, inhibition of RAS by ACE inhibitors may have a favorable effect on insulin action.

In recent years, an intrinsic RAS has been described in the pancreas of several species (Leung et al, 2004), with expression of angiotensin in the pancreatic islets, demonstrating a reduction in oxidative stress by the administration of ACE inhibitors, protecting the pancreatic islets from glucotoxicity generated by the presence of high glucose concentration. Lupi et al, (2019) observed that RAS molecules, such as ACE, AT1 and Ang II receptors, have increased expression when isolated pancreatic islets are cultivated in high glucose concentration, showing a reduction in insulin secretion and an increase in oxidative stress, as an increase in the concentration of nitrotyrosine and in the expression of protein kinase Cβ and NADPH oxidase. The presence of ACE inhibitors, such as zofenoprilat and enalaprilat, inhibited several of the deleterious effects of exposing pancreatic islets to high glucose concentration by reducing oxidative stress. In conditions that lead to beta cell damage, such as elevated blood glucose levels for prolonged periods or even prolonged exposure of beta cells to pro-inflammatory cytokines, which are common stimuli in diabetes, upregulated production and activity of NADPH oxidase can result in an excessive production of ROS in pancreatic beta-cells (De Ciuceis et al, 2005; Piro et al, 2002) In fact, Morgan et al. demonstrated that in isolated rat islets incubated in Krebs medium with high concentration of glucose and palmitic acid, the production of ROS via NAD (P) H oxidase is exacerbated.

In our model, the histological changes in the pancreatic islets of rabbits resulted from treatment with alloxan, with a tendency towards better protection of the pancreatic islets by marking with insulin in the group of non-diabetic rabbits treated with ACE inhibitors. In a study with diabetic rats, Tikellis et al. demonstrated a protective effect by using RAS blockers, both ACE inhibitors and AT1 receptor blockers, decreasing fibrosis in pancreatic islets (Tikellis et al, 2004) In a model of type 2 diabetes with rats, characterized by obesity and insulin resistance, olmesartan and temocapril attenuated the morphological changes in the pancreatic tissue.

Although we have observed in isolated pancreatic islets treated with ACE inhibitors, lower marking for oxidative stress, we were unable to demonstrate, through histological and immunohistochemical evaluation for insulin, protection in the pancreas of animals treated with ACE inhibitors. We only observed islet destruction by alloxan treatment, as ACE inhibitors were administered when the islet beta cells had already been destroyed by alloxan. Protective effect of ACE inhibitors may be better evidenced in models of type 2 diabetes, acting to prevent long-term deterioration of the pancreatic islet, delaying the onset of diabetes. Blocking the RAS can contribute to the development of new therapeutic strategies in the prevention and treatment of patients with diabetes.

Conclusion

The ACE inhibitor impaired the production of superoxide in islets isolated from rabbits, mainly in the presence of normal concentration of glucose, suggesting that the blockade of RAS may attenuate the deleterious actions of Ang II on pancreatic islet structure.

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Conflict of interest

None.

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