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Dipeptidyl peptidase IV inhibitor improves the salivary gland histology of spontaneously diabetic mice

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ABSTRACT

Objectives: The incretin-based therapy might be effective in patients possessing certain levels of preserved pancreatic beta-cells. However, doubts still exist regarding the efficacy of this atment in the recovery of tissues damaged by type 1 diabetes. Thus, the objective of this study was to evaluate the treatment with MK0431 in salivary glands of spontaneously diabetic mice, focusing mainly on the possible therapeutic and hypoglycaemic effects of this dipeptidyl peptidase IV inhibitor in the recovery of these salivary tissues.

Methods and results: Twenty mice were divided into two groups of 10 animals each: group I (NOD diabetic/untreated) and group II (NOD diabetic MK0431/treated). The group II was treated during 4 weeks with MK0431 mixed in the food. The group I was maintained in the same way without receiving, however, any treatment. Glucose levels were monitored during treatment and salivary glands samples were collected at the end of treatment for the histological examination under both transmitted and polarized light microscopy. High glucose levels were observed in untreated animals, while in animals with treatment, reduction of these levels was observed. Tissue restructuring was also observed in animals submitted to therapy with MK0431, mainly in relation to the attempt to extracellular matrix reorganization.

Conclusions: According to results, the treatment with this dipeptidyl peptidase IV inhibitor contributed to the general homeostasis of the organism and to the reestablishment of both epithelial and stromal compartments which were damaged by the hyperglycaemic condition, demonstrating that the incretin-based therapy may be an important complementary treatment for the type 1 diabetic condition.

1. Introduction

The diabetes mellitus consists of a group of metabolic disorder with common characteristics; the hyperglycaemia and the gluconeogenesis.[1,2] This disease affects approximately 10% of the diabetic patients in the occident, being one of the most frequent chronic diseases in infants, becoming a challenge to the public health.[3] Estimates show in 2030, that the prevalence will be 4.4% of people with diabetes in the world.[4] In Brazil, the diabetes affects around 11% of the adult population.[5]

Type 1 diabetes is related to immunological, environmental, and genetic factors, that cause the destruction of pancreatic beta-cells.[1,6] This disease affects the pancreas

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and can affect also different tissues and organs, including the salivary glands. Different studies describe the effects of diabetes mellitus in these glands. The authors describe cellular alterations and inflammatory process with the presence of CD3 cells. These complex harmful effects can compromise also the function of salivary tissues.[7–9]

Thus, the attempt of reversion of these alterations has been described in the literature. In this aspect, the treatments with incretins are related with the glucose homeostasis, insulin secretion and the inhibition of glucagon secretion. However, these hormones are quickly degraded by the action of the dipeptidyl peptidase IV (DPPIV) diminishing this possible therapeutic activity.[10–15] An option to avoid this degradation would be to use an incretin mimetic, as the MK0431. The function of this incretin mimetic is to inhibit the action of DPPIV, thus improving the glycaemic control by prolonging the action of glucagon-like peptide-1 (GLP1) and gastric inhibitory polypeptide (GIP). Moreover, the MK0431 can still stimulate the recovery and the maintenance of pancreatic cells.[12–14,16–19]

The salivary gland may be considered similar to pancreas in some aspects.[20] Accordingly, there is evidence indicating a relationship between insulin production and the salivary tissues. Although the salivary glands are typically exocrine, He et al. demonstrated endocrine secretions related to these tissues. Sánchez García et al., observed that insulin levels found in saliva were similar to plasma levels under normal conditions and suggested that the insulin might be a product of the salivary glands.[21,22] Thus, knowing this relationship between salivary glands and pancreas, the therapy with MK0431 can lead as yet to the recovery of salivary tissues, similar to the observed in pancreatic cells. However, doubts still exist regarding the efficacy of this treatment in recovery of tissues damaged by type 1 diabetes. Therefore, this study evaluated the treatment with MK0431 in salivary glands of spontaneously diabetic mice, focusing mainly on the possible therapeutic and hypoglycaemic effects of this dipeptide peptidase IV inhibitor in the recovery of these salivary tissues.

2. Materials and methods

2.1. Animals and tissues

Twenty 15-week-old female NOD mice, weighing on average 25 g, were divided into two groups: 10 diabetic NOD mice (group I) and 10 also diabetic NOD mice (group II). The animals were obtained from the Animal House of State University of Campinas (CEMIB-UNICAMP) and were kept under standard conditions of housing, feeding and treatment at the Sector of Laboratory Animal Experimentation (SEA), Department of Morphology and Basic Pathology, Faculty of Medicine of Jundiaí, Brazil. Blood glucose (mg/dL) was measured weekly in all animals with a blood glucose meter (Accu-Chek Performa, Roche, Switzerland). After characterization of the diabetic condition, animals of both groups presented glucose levels higher than 300 mg/dL.[23]

Then, the animals of group II received MK0431 mixed in pelleted diet (11 g/kg) similar to Lamont and Drucker[24] for a period of 4 weeks.[17] In order to simulate the experimental conditions of treated group, animals of the group I were manipulated in the same way and received pelleted diet and water *ad libitum*, however, without hypoglycaemic agents.

After treatment, the animals were anaesthetised (imp.) with a mixture of ketamine hydrochloride (130 mg/kg, Francotar, Virbac, Brazil) and xylazine hydrochloride (6.8 mg/kg, 2% Virbaxyl, Virbac, Brazil) and salivary gland samples were collected for analyses by transmitted and polarized light microscopy. Then, the animals were euthanized with an overdose of the anaesthetic according to the ethical guidelines of the Brazilian College of Animal Experimentation (COBEA). In all animals, the proteinuria (mg/dL) and urine pH were analysed and the body weight (g) was evaluated at the beginning and at the end of the experiment.

Samples of parotid and submandibular salivary glands were fixed in Bouin's solution (picric acid solution), embedded in plastic resin (Paraplast Plus, Oxford Lab, MO, USA), and stained with hematoxylin-eosin (H.E.). Parts of these samples were also stained with Picrosirius red (saturated aqueous solution of picric acid supplemented with 0.1 g Sirius red F3b dye content 25%; Bayer, Germany) for analysis of extracellular matrix fibrillar components by polarized light microscopy.

2.2. Transmitted and polarized light microscopy

2.2.1. Stereology – three-dimensional analysis of tissues

The nuclear and cytoplasmic volumes of the acinar cells of parotid and submandibular glands were determined in H.E. – stained histological sections by transmitted light microscopy. For this purpose, 50 cells were analysed per animal, for a total of 500 acini per experimental group, by the point counting method described by Weibel.[25] Only intact cells and circular or ellipsoid nuclei with defined limits were considered for this study. In addition, collagen fibres and the spatial volume density of these components were analysed under polarized light and calculated as the mean of ten regions in each Picrosirius-red-stained histological section also by the point-counting method.[26,27] Moreover, the relative area occupied by epithelium and glandular stroma was measured with the Image J 1.39 image analysis system (Image Processing and Analysis in Java, National Institutes of Health, Maryland, USA).

All analyses were performed with a Nikon Eclipse microscope using 10×, 40× and 100× planachromatic objectives for transmitted light microscopy and birefringent lenses for polarized light microscopy. The microscope was coupled to the SD-3.3 CCD image acquisition system of the Department of Morphology and Basic Pathology, Faculty of Medicine of Jundiaí.

2.3. Statistical analysis

The results are reported as the mean ± standard deviation for determination of body weight variation (g/final weight – ini-initial weight) and glucose levels (mg/dL) by analysis of variance (ANOVA), and as the median for nuclear and cytoplasmic volume of acinar cells of the parotid and submandibular salivary glands (μm^3), relative area of secretory epithelium, relative area of glandular stroma, and volume density of collagen fibres (%), by the non-parametric Kruskal–Wallis test for pairwise comparison.[28] The level of significance was set at 5% for all tests.

Table 1 – Body weight variation (final weight − initial weight) in the groups studied.

	Weight variation (g)
Group I (untreated)	−1.20 ± 0.72
Group II (treated)	−0.65 ± 0.45*

Values are expressed as the mean ± SD.
* Not significantly different ($P > 0.05$).

Table 2 – Glucose levels of treated animals and untreated animals during the experimental period.

	Glucose levels (mg/dL)
Group I (untreated)	521.1 ± 103.50
Group II (treated)	349.0 ± 183.76*

Values are expressed as the mean ± SD.
* Significantly different ($P < 0.05$).

3. Results

3.1. *Body weight variation*

All animals demonstrated weight loss after establishment of the diabetic condition. In treated group, it was not observed body weight gain (Table 1).

3.2. *Proteinuria and urinary pH*

In animals of group II, urine pH ranged from 6 to 7.5 and the protein levels were 7.5 mg/dL. In contrast, animals of group I presented an average urine pH of 5.0–7.0 and the proteinuria levels were 100 mg/dL, indicating an uncontrolled diabetic state.

3.3. *Glucose levels*

Animals of group I presented elevated blood glucose levels, thus maintaining the diabetic state throughout the experimental period. In contrast, a significant reduction of the glucose levels was observed in animals of group II treated with MK0431 (Table 2).

3.4. *Transmitted and polarized light microscopy*

3.4.1. *Parotid glands*

In animals submitted to the therapy with MK0431 (group II) it was observed involuted serous acini. However, a recovery was noted when compared to untreated animals (Fig. 1A and Tables 3 and 4). Stromal spaces filled with extracellular matrix were identified between acini by Picrosirius red staining. The quantity of collagen fibres was significantly minor than that observed in untreated animals (Fig. 1C and Table 5).

Pleomorphic serous acini characterized by a reduced spatial area occupied by secretory epithelium were observed in parotid glands of the group I (Fig. 1B and Tables 3 and 4). The stroma was found enlarged, with a higher volume density of collagen fibres (Fig. 1D and Table 5).

3.4.2. *Submandibular glands*

Similarly, animals submitted to therapy with MK0431 (group II) presented also involuted seromucous acini; however, a significant recovery was also noted when compared to untreated animals (Fig. 2A and Tables 3 and 4). In the same way, stromal spaces filled with extracellular matrix were

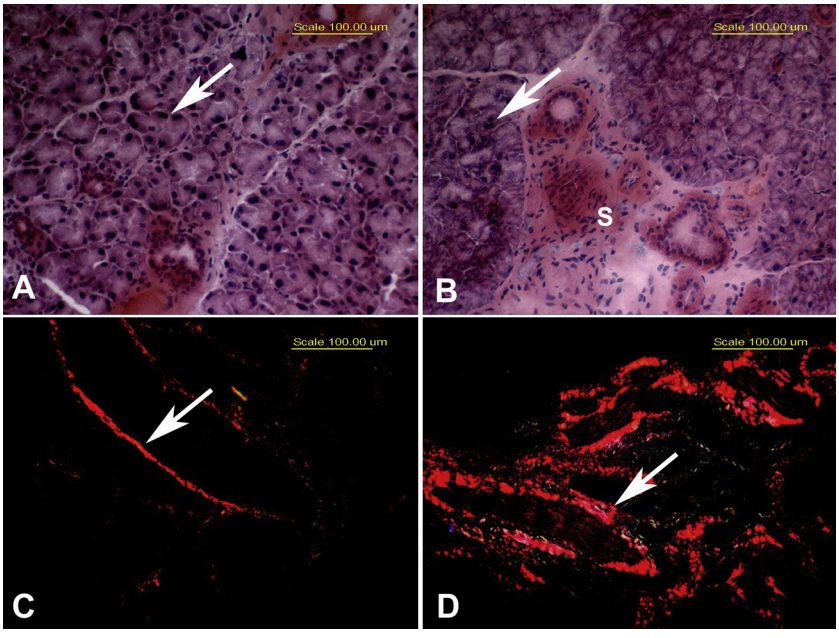

Fig. 1 – Photomicrograph of the parotid gland. (A) Group II presented serous acini recovered (arrow). (B) Group I presented pleomorphic serous acini (arrow) and increase of area occupied by stroma (S). H.E. (C) In group II, stromal spaces filled with extracellular matrix were identified between acini (arrow). (D) In animals untreated, the stroma was found enlarged, with a higher volume density of collagen fibres (arrow). Picrosirius red.

Table 3 – Nuclear and cytoplasmic volumes of the parotid and submandibular glands in the groups studied.

Group	Parotid gland		Submandibular gland	
	Nuclear volume (μm^3)	Cytoplasmic volume (μm^3)	Nuclear volume (μm^3)	Cytoplasmic volume (μm^3)
I (untreated)	27.65*	23.45*	32.69*	26.20*
II (treated)	48.10*,a	103.97*,b	54.26*,a	121.84*,b

Values are expressed as the median. Same superscript letters indicate significantly different ($P < 0.01$).
* Significantly different ($P < 0.01$).

Table 4 – Relative area (%) of the secretory epithelium versus glandular stromal area in the parotid and submandibular glands of treated or not treated animals with MK0431.

Groups	Parotid gland		Submandibular gland	
	Epithelial area	Stromal area	Epithelial area	Stromal area
I (untreated)	29.21*	70.79*	25.12*	74.88*
II (treated)	38.02*,a	61.98*,b	39.22*,a	60.78*,b

Values are expressed as the median. Same superscript letters indicate significantly different ($P < 0.01$).
* Significantly different ($P < 0.01$).

identified between acini by Picrosirius red staining. The quantity of collagen fibres was significantly minor (Fig. 2C and Table 5).

In submandibular glands, atypical and involuted seromucous acini were observed in the group I (Fig. 2B and Tables 3 and 4). Enlargement of the interacinar spaces were also observed. Extracellular matrix alterations were observed in the stroma, with the observation of increase in the connective tissue component (Fig. 2D and Table 5).

4. Discussion

Diabetic animals presented the lowest weight throughout the experimental period. Diabetes mellitus causes metabolic disorders and body weight alteration.[29–32] Animals submitted to glycaemic treatment, showed recovery of body weight.[33] Body weight recovery and gain were observed also after use of incretin-based therapies; however, this cannot reflect an adequate metabolic control. An alternative to the diabetes treatment and weight control is the use of DDPIV inhibitors, as the sitagliptin (MK0431). This incretin mimetic promotes the maintenance and in different cases the loss of weight, as observed in both type 1 and 2 diabetes.[14,17,18,34–36]

Therefore, while weight gain can exacerbate hyperglycemia, the minor weight observed in treated animals may be related to the beneficial effect of treatment with this DPPIV inhibitor.

As per to glucose levels, it was observed elevated levels throughout the experimental period in animals of group I, and a significant reduction of glucose levels was observed in animals of group II. In a study using insulin replacement therapy, a proven hypoglycaemic treatment, Hu et al. [37] showed that normal glucose levels in healthy animals are close to 180 mg/dL, whereas mean levels of 300 mg/dL or higher indicate an effective diabetic state.[23]

The reduction of glucose levels after treatment with MK0431 can be associated with the recuperation of mass and function of pancreatic beta-cells. In an experimental study, this significant reduction in the glucose levels was also confirmed.[16,38] Contrariwise, Kim et al. observed not in type 1 diabetes significant alteration in glucose levels after administration of MK0431.[17] These results show that the potential relation of incretins or incretin mimetics and the type 1 diabetes remain unclear. Anyway, Gutniak et al. and Creutzfeldt et al. provide evidence to support the potential utility of incretin-based therapies in the treatment of diabetes mellitus.[38–40]

Lastly, our data also permit to conclude that the animals presented an effective diabetic condition and the therapy with MK0431 played an important role in the reduction of hyperglycemia condition.

The relation existing between salivary glands and pancreas has been described in literature. Some authors also demonstrated that these organs may share a common antigen that might be the target of the autoimmune process in the type 1 diabetes.[41]

Similarly, in the present study, the salivary glands of diabetic animals were target of this hyperglycaemic condition, presenting structural changes, characterized by pleomorphic acini, minor spatial area occupied by secretory epithelium and a higher volume density of collagen fibres. In contrast, recovery of the glandular structure was observed in the group treated with MK0431. The submandibular glands of treated animals presented a higher recovery, characterized by a minor quantity of collagen fibres and organization of secretory epithelial cells. This positive finding might be explained by physiological and anatomical similarity between the submandibular gland and

Table 5 – Median variation in the volume density of collagen (%) in the parotid and submandibular gland of treated (group II) and untreated animals (group I).

Parotid gland	Collagen fibres
Group I	45.05[a]
Group II	21.29[a,*]
Submandibular gland	**Collagen fibres**
Group I	30.80[b]
Group II	15.10[b,*]

Same superscript letters (a,b) indicates significantly different ($P < 0.01$).
* Significantly different ($P < 0.01$).

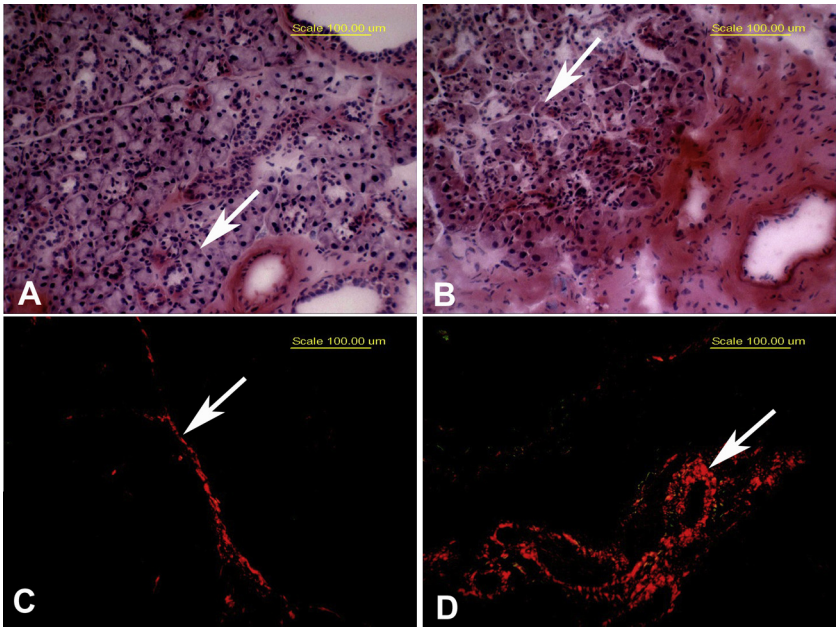

Fig. 2 – Photomicrograph of the submandibular glands. (A) In animals submitted to MK0431 therapy (group II), were noted seromucous acini recovered (arrow). (B) Atypical and involuted seromucous acini were observed in the group I (arrow). H.E. (C) Group II presented stromal spaces filled with extracellular matrix (arrow). (D) Increase of extracellular matrix was observed in the stroma of untreated animals (arrow). Picrosirius red.

pancreas,[42–44] thus responding better to the treatment with MK0431.

The morphological characteristics of salivary glands in healthy animals, the relationship between these normal tissues and incretins, and the effects of diabetes on these organs have been documented.[7,45,46] Simões et al. observed the accumulation of lipid droplets in the glands of hyperglycaemic rats, elements characteristic in processes of tissue damage.[47] Also, alterations in saliva components were observed in salivary glands of diabetic animals and the tissue responses to this condition were different when compared to the mucous and serous glands.[48,49] Additionally, the effects of diabetes have also been described in salivary glands of humans, characterized by small acini, a bigger number of lipid intracytoplasmic droplets in the acinar and ductal cells, increased volumes of fibrous tissue, as well as an abundant adipose infiltration in the stroma.[41,50,51]

To reverse these damages, treatments with incretins and incretin mimetics can be an important tool in recovering of tissues. However, despite of promising results, the MK0431 can be related also to cellular complications and doubts still exist regarding the total efficacy of this treatment in different cases.[52,53] Thereby, further studies will be necessary to better understand the mechanism underlying the action of this incretin mimetic.

Anyway, the treatment with this dipeptidyl peptidase IV inhibitor contributed to the general homeostasis of the organism and to the reestablishment of both epithelial and stromal compartments which were damaged by the hyperglycaemic condition, demonstrating that the incretin-based therapy may be an important complementary treatment for the type 1 diabetic condition.

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Competing interests

None declared.

Ethical approval

This study was approved by the Brazilian College of Animal Experimentation (COBEA) and the Institutional Ethics Committee (180/10).

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