Role of GAD peptides p217 and p290 in the repair of INS receptor in salivary tissues of type 1 diabetic mice

Rodrigo Eduardo da Silva[1], Nilva de Karla Cervigne[1], Clovis Antonio Lopes Pinto[2], Marcelo Rodrigues da Cunha[1], Fernanda Rojas[1], Luis Antonio Peroni[1], Taize Machado Augusto[1], Evaldo Marchi[3], Barkat Ali Khan[4] and Eduardo José Caldeira[1]*

[1]Tissue Morphology Laboratory, Department of Morphology and Basic Pathology, Faculty of Medicine of Jundiaí, São Paulo, Brazil
[2]Pathology Laboratory, Department of Morphology and Basic Pathology, Faculty of Medicine of Jundiaí, Jundiaí, São Paulo, Brazil
[3]Surgery Department, Medical College of Jundiaí, FMJ, Jundiaí, São Paulo, Brazil
[4]Faculty of Pharmacy, Gomal University, D.I Khan, KPK, Pakistan

Abstract: Glutamate decarboxylase or glutamic acid decarboxylase (GAD) is a protein associated with autoimmune diseases, including type-1 diabetes. This disease is primarily associated with the occurrence of a specific isoform: GAD65. Conversely, some specific peptides of this protein may block autoimmunity in diabetes. In this respect, understanding the relationship between GAD and the development of diabetes is important, and it is necessary to understand the role of each GAD peptide to design effective autoimmune diabetes treatments. The purpose of the present study was to analyze the effects of treatment with GAD-derived peptides p217 and p290 on INS receptors in the salivary epithelium of nonobese diabetic (NOD) animals. Three groups of 7 mice each were studied: I, BALB/c mice (control); II, NOD mice; and III, NOD mice treated with peptides p290 and p217. Groups I and II only received buffered saline solution. Glucose levels were measured daily during the 21 days of the experiment. After the study, the animals were euthanized and the parotid and submandibular glands were removed for the analysis of INS-R by fluorescence microscopy. Therapy with two peptides together was associated with reduced glucose levels in NOD mice and intense INS-R expression in both salivary organs. Our approach of combining GAD p217 and p290 peptides contributed to hormonal balance and promoted the repair of INS-R.

Keywords: Glutamic acid decarboxylase, insulin, insulin receptor, nonobese diabetic mice.

INTRODUCTION

Like many other organ systems, the salivary glands are targets of insulin (INS) and are affected by diabetes, which causes numerous complications (Fushini, 1980; Ho, 1990; Cagnon *et al.*, 2000; Chavez *et al.*, 2000; Carvalho *et al.*, 2003; Pace *et al.*, 2003; Shirai *et al.*, 2004; Caldeira and Cagnon, 2008; Kumar, 2004). INS is related to cellular maintenance, and its action depends on its ability to bind its receptors (Ullrich *et al.*, 1985; Goldfine, 1987; White and Kahn 1994; Caldeira and Cagnon, 2008). INS receptors (INS-R) are heterotetrameric proteins comprised of alpha and beta subunits with intrinsic kinase activity, and first interaction between INS and its receptor requires the alpha subunits (Patti and Kahn, 1998).

Type 1 diabetes affects glandular organs and their homeostasis. This disease is initiated by chronic inflammation, leading to pancreatic beta-cell damage that results in general INS deficiency and hyperglycemia. Similarly, tissue damage affecting the salivary glands is also mediated by an autoimmune process primarily involving cytotoxic T cells. Autoantibodies against INS, the protein tyrosine phosphatase (ICA 512), and glutamic acid decarboxylase (GAD) also play roles in these detrimental processes (Karlsson *et al.*, 2000; Fenalti and Buckle, 2010; Metidieri *et al.*, 2012).

Glutamic acid decarboxylase (GAD) is an autoantigen of INS-secreting cells that is expressed in both human patients and nonobese diabetic (NOD) mice (De Aizpurua *et al.*, 1994; Pleau *et al.*, 1995; Yoon *et al.*, 1999). GAD is primarily expressed in the nervous tissue, but this protein is also found in other tissues, including the pancreas. However, the relationship of GAD with this glandular organ is not entirely clear (Petersen *et al.*, 1998; Esclapez and Houser, 1999; Wei and Wu, 2008; Ludvigsson, 2009).

In the last few years, considerable progress has been made in describing and characterizing GAD autoantigens. Several isoforms of GAD have been reported, including GAD 65 (Roep, 1996; Ludvigsson *et al.*, 2008; Fenalti and Rowley, 2008). Despite the relationship of GAD with autoimmune processes, recent studies have suggested that GAD and its peptides can prevent the progression of diabetes (Liu, 2006). GAD protein therapy has been shown to induce an immune tolerance and, as a consequence, can potentially interrupt pancreatic cell destruction (Morales and Thrailkill, 2011). In contrast, other studies have demonstrated that GAD can increase the inflammatory process in models of experimental diabetes (Gauvrit *et al.*, 2004). These conflicting results

*Corresponding author: e-mail: drdcaldeira@gmail.com

illustrate the complexity of GAD and the need for additional investigations. Such studies are important, as this approach could be a potential therapy to reverse the damage that diabetes causes in salivary gland epithelial cells.

Therefore, the purpose of this work was to analyze the effects of treatment with the GAD-derived peptides p217 and p290 on INS receptor expression in the salivary epithelium of NOD mice.

MATERIALS AND METHODS

Animal protocol - We obtained 15-week-old mice (females/ n=21/ weighing 20±2.5g) from the Animal Care Centre (CEMIB) of Universidade Estadual de Campinas. The animals were divided in three groups (n=7 each): I, BALB/C control mice; II, NOD mice without treatment; and III, NOD mice treated with GAD peptides p217 and p290. The animals were maintained under standardized conditions at the Animal Experimentation Laboratory, Faculty of Medicine of Jundiaí, FMJ, São Paulo, Brazil.

Groups I and II received 1-ml intraperitoneal injections of phosphate-buffered saline (PBS) alone to emulate the experimental phases. After hyperglycemia was confirmed in group III, each animal received an intraperitoneal injection of 1 ml PBS plus GAD peptides p217 (200µg) associated with p290 (200µg) (peptides purity >98%) (Rheabiotech, São Paulo, Brazil) diluted in IFA (incomplete Freund′s adjuvant/100µl). During the 21-day experiment, the animals received three intraperitoneal injections, the first one 7 days after disease confirmation, and then successively on days 14 and 21, similarly to what was previously described (Tisch *et al.*, 1999).

The blood glycaemia levels (mg/dl) were observed on a weekly basis with Accu-Chek test strips (Performa, Roche Diagnostics, Basel, Switzerland). The mean glucose level of control animals was 169mg/dl. Diabetes was defined as values 300mg/dl or higher (Shirai *et al*., 1998). Following the experimental period, animals were anesthetized with ketamine (Ketalar, Parke-Davis/Pfizer, New York, NY, USA) and xylazine (Rompun, Bayer Animal Health, Shawnee, KS, USA) at a dose of 0.02g/ml. When the animals were completely anesthetized, the salivary glands were extracted by surgical procedure. The rodents were then sacrificed with a lethal dose of anesthetics, in accordance with ethical guidelines for animal handling.

Immunofluorescence microscopy

Immunohistochemistry is an important tool used for the diagnosis and prognosis of multiple cases, and it is frequently used to detect the localization of proteins in tissue sections. Salivary gland specimens were cut in 8-µm-thick slices and included in blocking solution for 1 h at ambient temperature. The specimens were then incubated for 12 h (overnight) with the primary antibody raised against INS-R (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then cleaned with PBS, and included for 2 h with secondary fluorescent antibody (goat anti-rabbit, Santa Cruz Biotechnology). The slices were then cleaned in PBS and mounted in DABCO (Sigma, St. Louis, MO, USA) for microscopic analysis.

The samples were observed under an immunofluorescence microscope (Leica, Wetzlar, Germany) at the Faculty of Medicine of Jundiaí. The images were acquired using a 10× objective lens. A subset of specimens in which the primary antibody was omitted served as negative controls. The immunostaining was graded using a semi-quantitative method and scored as mild, moderate, or saturated, according to INS-R labeling intensity and distribution as previously reported (Markopoulos *et al.*, 2000; Caldeira and Cagnon, 2008).

RESULTS

Glucose levels

Diabetic animals (group II) had serum glucose levels above 660mg/dl. A mean lower glucose level (485mg/dl) was observed in the treated group (III). The mean glucose level in nondiabetic control mice (group I) was 169mg/dl.

Immunofluorescence microscopy

Submandibular gland

In control animals (group I), a saturated INS-R signal was observed (fig. 1A and table 1). In group II (NOD mice without treatment), receptor labeling was mild, with localization in salivary epithelium (fig. 1B and table 1). In group III, however, INS-R labeling was also saturated and localized in the salivary epithelium, as noted in group I (fig. 1C and table 1).

Parotid gland

Similarly, in parotid tissues of group I (control animals) high INS-R levels were observed in the salivary epithelium (fig. 1D and table 1). The INS-R labeling in untreated animals of group II was mild, with similar localization (fig. 1E and table 1). Similar to what was found in the submandibular gland, INS-R labeling in diabetic mice (group III) was intense in the salivary epithelium (fig. 1F and table 1).

DISCUSSION

NOD mice are a typical model for studying type 1 diabetes and INS effects (Leiter, 1989). Although INS is mainly produced by the pancreas, it may be produced by other tissues. This hormone modulates physiological events in many tissues, including the salivary glands. It is known that INS controls glucose levels and maintains

cellular energy balance (Kerr *et al.*, 1995; Katz *et al.*, 2003; Cunha *et al.*, 2007; Volp *et al.*, 2008). According to Hu *et al*. (1992), normal serum glucose levels in mice are 180 mg/dl, and a diabetic state is defined when the mean glucose level is higher than 300mg/dl (Shirai *et al.*, 1998). These values were used to assess the diabetic state of the animals in our study. Our findings show that treatment with GAD peptides p217 and p290 successfully reduced glycemic levels in NOD mice.

The immunofluorescence analysis revealed alterations in INS-R levels in diabetic animals. In contrast, receptor expression was normalized in the treated group, to a level similar to that observed in healthy mice. Others factors, as well as hormones and peptides, can affect cellular homeostasis, including in salivary tissues. Scientific evidence from animal models indicates that changes in these hormones, proteins and their receptors may lead to salivary gland pathology. Several studies have confirmed the influence of these membrane receptors (mainly INS-R) on cellular homeostasis (Caldeira and Cagnon, 2008; Gorjup *et al.*, 2009; Maekawa *et al.*, 2011; Yashida *et al.*, 2011; Csete and Doyle, 2014). Similar findings were observed in the present experiments, suggesting that treatment with GAD peptides led to an increase in INS and modulation of its receptors. In a previous study, we demonstrated that diabetes altered INS-R expression in animals in which glycemic control was achieved with INS treatment (Caldeira and Cagnon, 2008). This finding suggests that there were changes in the production of INS and the interaction of INS with the alpha subunits of INS-R. This may indicate an affinity between endocrine INS and the salivary glands, similar to what occurs in tissues in subjects with type 2 diabetes. This result is in accordance with findings reported for types 1 and 2 diabetes.

Importantly, we found that peptide-based therapy led to INS-R recovery. These results are similar to those observed in previous studies in which experimental treatment with GAD or its peptides could prevent diabetes progression. These authors also emphasized that the peptides p217 and p290 of GAD65 may block the damage to the pancreas caused by hyperglycemic conditions. This process is probably related to the deletion of antigen-specific pathogenic T cells, thus impeding the progression of autoimmune diabetes (Tisch *et al.*, 1999; Chen *et al.*, 2003; You *et al.*, 2004; Liu, 2006).

In autoimmune diabetes, the activation of CD4+ T-lymphocytes promotes cellular destruction through the action of cytotoxic CD8+ T-lymphocytes. In the second stage of pathogenesis, discharge of cytokines by CD4+ T-lymphocytes promotes the formation of oxygen free radicals that maintain tissue destructive inflammatory processes (Calcinaro *et al.*, 1996). GAD65 is a sensitive marker for these events, indicating an autoimmune attack on pancreatic beta cells.

Conversely, the GAD65 protein can protect diabetic animals from this immune destruction, indicating that GAD65 is also important for the maintenance and control of the autoimmune response (Yoon *et al.*, 1999). Despite this, other authors have noted that therapy with GAD and its peptides is only effective in animal models, and the treatment of human autoimmune diseases remains a challenge (Chen *et al.*, 2003; Gauvrit *et al.*, 2004; You *et al.*, 2004; Wherrett *et al.*, 2011).

Another study demonstrated that the GAD peptides p217 and 290 could be fused to other molecules. These fusion proteins can maintain peripheral cellular structures, suggesting new possibilities for the functional recovery of these components (Wang *et al.,* 2009). The approach used in our study may have promoted a fusion between the peptides and the alpha subunits of INS-R, thus restoring the interaction between INS and its receptor.

CONCLUSION

These data strongly suggest that GAD peptides (p217 and p290) are good targets of treating type 1 DM. Thus, therapies that are directed at these peptides response might be of benefit in controlling type 1 diabetes, which is supported by studies that show combining GAD p217 and p290 peptides contributed to hormonal balance and promoted the repair of INS-R in NOD mice. Furthermore, these findings suggest that GAD specific CD4+T-cell promiscuity reflects a novel form of T-cell avidity maturation Our results also suggest that these fusion proteins may be effective in inhibiting the development of diabetes and promoting the recuperation of INS receptor expression in salivary tissues. This treatment contributed to lower glucose levels and possibly repaired hyperglycemia-induced damage to salivary glands, indicating that this peptide combination may be therapeutically effective. Additional studies are needed to elucidate the mechanisms underlying the benefits of the GAD-derived p217 and p290 peptides in relation to glucose control.

ACKNOWLEDGMENTS

This work was supported by the Nucleus of Support to Research and Teaching (NAPED) of the Faculty of Medicine of Jundiaí, SP. Brazil, and Research Foundation of the State of São Paulo (FAPESP) (grant number: 2012/18012-2).

ABBREVIATIOS

GAD: Glutamic Acid Decarboxylase, NOD: Nonobese diabetic, INS R: Insulin Receptor

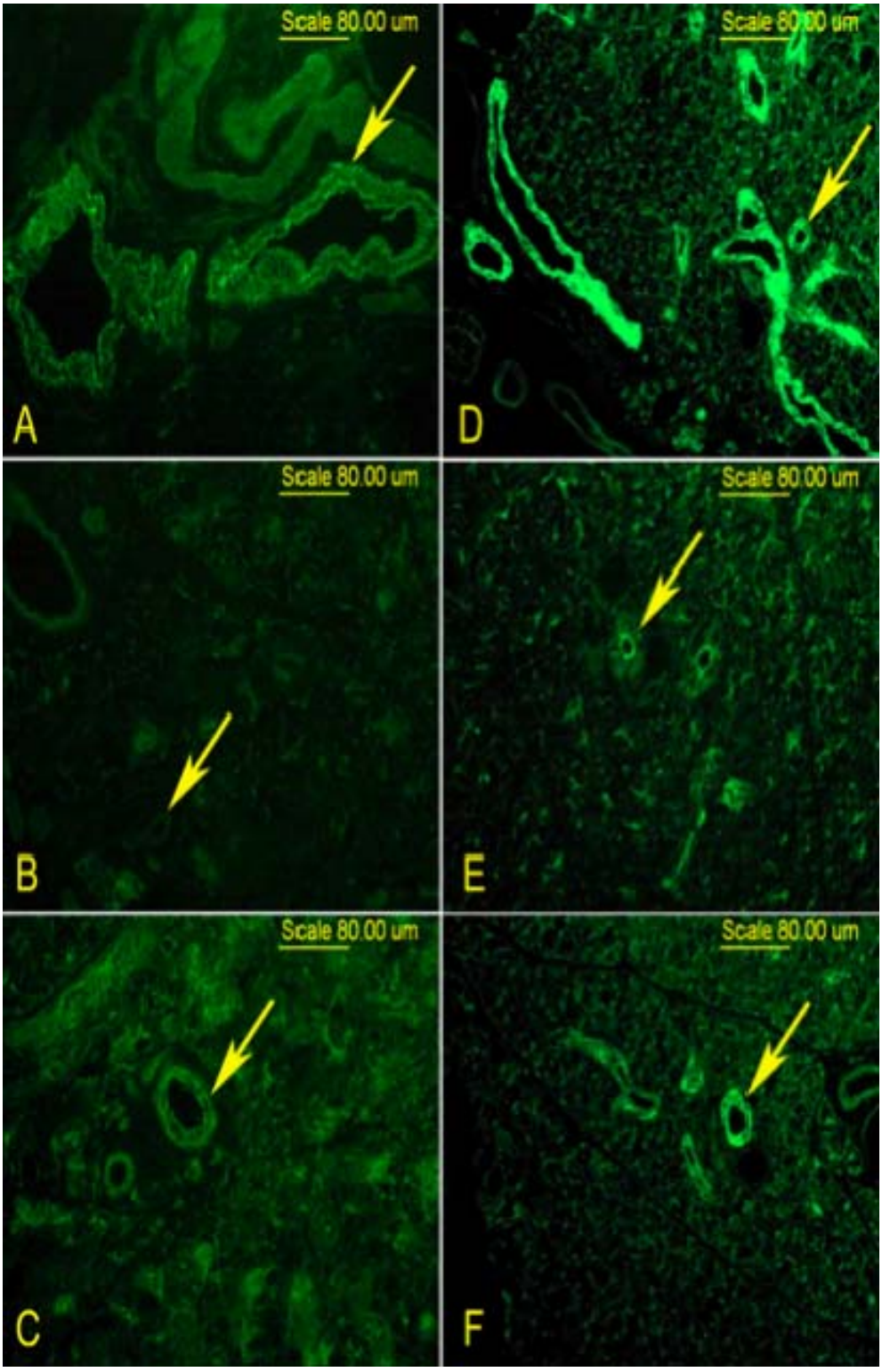

Fig. 1: Immunostaining of submandibular glands. A: Group I, showing saturated expression of INS-R (arrow). B: Group II, showing mild INS-R expression (arrow). C: NOD mice treated with peptides p217 and p290 (Group III), showing saturated INS-R expression similar to control non-diabetic mice (arrow). Immunostaining of the parotid glands. D: Group I, with saturated expression of INS-R (arrow). E: Group II, with mild INS-R expression (arrow). F: Treated animals of group III, showing saturated INS-R expression similar to control non-diabetic mice (arrow).

Table 1: Semi-quantitative evaluation of the INS-R expression in salivary glands of control non-diabetic mice (group I) and NOD mice without treatment (group II) or treated with GAD peptides p217 plus p290 (Group III).

Groups	Submandibular gland	Parotid gland
	INS-R	INS-R
I	+++	+++
II	+	+
III	+++	+++

+ mild; ++: moderate; +++: saturated.

REFERENCES

Cagnon VH, Camargo AM, Rosa RM, Fabiani R, Padovani CR and Martinez FE (2000). Ultra structural study of the ventral lobe of the prostate of mice with streptozoticin induced diabetes (C57BL/6J). *Tissue Cell*, **32**: 275-283.

Calcinaro F, Lafferty KF and Shehadeh NN (1996). Inflammatory mediators and development of autoimmune diabetes. In: Eisenbarth G, Lafferty KJ, editors. Type I diabetes. Cary, Oxford University Press, pp.91-118.

Caldeira EJ and Cagnon VH (2008). IGF-I and INS receptor expression in the salivary glands of diabetic Nod mice submitted to long-term insulin treatment. *Cell Biol. Int.*, **32**: 16-21.

Carvalho CAF, Camargo AM, Cagnon VH and Padovani CR (2003). Effects of experimental diabetes on the structure and ultrastructure of the coagulating gland of C57BL/6J and Nod mice. *Anat. Rec. A Discov. Mol. Cell Evol. Biol.*, **270**: 129-136.

Chavez EM, Taylor GW, Borrell LN and Ship JA (2000). Salivary function and glycemic control in older persons with diabetes. *Oral Surg. Oral Med. Oral Pathol.*, **89**: 305-311.

Chen C, Lee WH, Yun P, Snow P and Liu CP (2003). Induction of autoantigen specific Th2 and Tr1 regulatoryT cells and modulation of autoimmune diabet es. *J. Immunol.,* **171**: 733-744.

Cunha DA, de Alves MC, Stoppiglia, LF, Jorge AG, Modulo CM, Carneiro EM, Boschero AC, Saad MJ, Velloso LA and Rocha EM (2007). Extra-pancreatic insulin production in rat lachrymal gland after streptozotocin-induced islet beta-cells destruction. *Biochem. Biophys. Acta.,* **1770**: 1128-1135.

Csete M and Doyle J (2014). The mathematician's control toolbox for management of type 1 diabetes. *Interface Focus*, **6**: 20140042.

De Aizpurua HJ, French MB, Chosich N and Harrison LC (1994). Natural history of humoral immunity to glutamic acid decarboxylase in non-obese diabetic (NOD) mice. *J. Autoimmun.*, **7**: 643-53.

Esclapez M and Houser CR (1999). Up-regulation of GAD65 and GAD67 in remaining hippocampal GABA neurons in a model of temporal lobe epilepsy. *J. Comp. Neurol.*, **412**: 488-505.

Fenalti G and Rowley MJ (2008). GAD65 as a prototypic autoantigen. *J. Autoimmun.,* **31**: 228-232.

Fenalti G and Buckle AM (2010). Structural biology of the GAD autoantigen. *Autoimmun. Rev.*, **9**: 148-152.

Fushini, H (1980). The effect of parabiosis on serum and kidney glicosidase activities is spontaneously diabetic mice. *Diabetologia*, **19**: 50-53.

Gauvrit A, Debailleul M, Vu AT, Sai P and Bach JM (2004). DNA vaccination enconding glutamic acid decarboxylase can enhance insulitis and diabetes in correlation with a specific Th2/3 CD4 T cell response in non-obese diabetic mice. *Clin. Exp. Immunol.*, **137**: 253-262.

Goldfine ID (1987). The insulin receptor: Molecular biology and transmembrane signalling. *Endocr. Rev.*, **8**: 235-255.

Gorjup E, Danner S, Rotter N, Habermann J, Brassat U, Brummendorf TH, Wien S, Meyerhans A, Wollenberg B, Kruse C and von Briesen H (2009). Glandular tissue from human pancreas and salivary gland yields similar stem cell populations. *Eur. J. Cell Biol.*, **88**: 409-21.

Ho SM (1990). Prostatic androgen receptor and plasma testosterone level in streptotocin induced diabetic rats. *J. Steroid Biochem. Mol. Biol.*, **38**: 67-72.

Hu Y, Nakagawa Y, Purushotham KR and Humphreys-Beher MG (1992). Functional changes in salivary glands of autoimmune disease-prone NOD mice. *Am. J. Physiol.*, **263**: E607-614.

Karlsson FA, Berne C, Björk E, Kullin M, Li Z, Ma JY, Schölin A, Zhao L (2000). Beta-cell activity and destruction in type 1 diabetes. *Ups J. Med. Sci.*, **105**: 85-95.

Katz J, Stavropoulos F, Cohen D, Robledo J, Stewart C and Heft M (2003). IGF 1 and Insulin receptor expression in the minor salivary gland tissues of Sjögren's syndrome and mucoceles-immunohistochemical study. *Oral Dis.*, **9**: 07-13.

Kerr M, Lee A, Wang PL, Purushotham KR, Chegini N, Yamamoto H and Humphreys-Beher MG (1995). Detection of insulin and insulin-like growth factors I and II in saliva and potential synthesis in the salivary glands of mice. *Biochem. Pharmacol.*, **49**: 1521-1531.

Kumar V, Abbas AK and Fausto N (2004). Robbins & Cotran Patologia, 7th ed. Elsevier, pp.1243-1262.

Leiter EH (1989). The genetics of diabetes susceptibility in mice. *FASEB. J.*, **11**: 2231-2241.

Liu CP (2006). Glutamic acid decarboxylase-specific CD4+ regulatory T cells. *Ann. NY Acad. Sci.,* **1079**: 161-170.

Ludvigsson J, Faresjö M, Hjorth M, Axelsson S, Chéramy M, Pihl M, Vaarala O, Forsander G, Ivarsson S, Johansson C, Lindh A, Nilsson NO, Aman J, Ortqvist E, Zerhouni P and Casas R (2008). GAD treatment and insulin secretion in recent-onset type 1 diabetes. *N. Engl. J. Med.*, **359**: 1909-1920.

Ludvigsson J (2009). Therapy with GAD in diabetes. *Diabetes Metab. Res. Rev.*, **25**: 307-315.

Maekawa ET, Maioral EE, Metidieri HT, Picardi PK and Caldeira EJ (2011). Recovery of INS-R and ER-alpha expression in the salivary glands of diabetic mice submitted to hormone replacement therapy. *Arch. Oral Biol.*, **56**: 1129-36.

Markopoulos AK, Poulopoulos AK, Kavavis I and Papanayotou P (2000). Immunohistochemical detection on insulin-like growth factor I in the labial salivary glands of patients with Sjogren's syndrome. *Oral Dis.*, **6**: 31-34.

Metidieri HT, Mancio RD, Mayoral EE, Rojas FA, Peroni LA, Ferri AT, Lourenço EA and Caldeira EJ (2012). Effects of anti-CD3 monoclonal antibody in salivary glands of spontaneously diabetic mice. *Microsc. Res. Tech*., **75**: 928-934.

Morales AE and Thrailkill KM (2011). GAD-alum immunotherapy in Type 1 diabetes mellitus. *Immunotherapy*, **3**: 323-332.

Pace AE, Nunes PD and Ochoa-Vigo K (2003). Family knowledge about the problematics of patients with diabetes mellitus. *Rev. Lat. Am. Enfermagem*, **11**: 312-319.

Patti ME and Kahn CR (1998). The insulin receptor: A critical link in glucose homeostasis and insulin action. *J. Basic Clin. Physiol. Pharmacol.*, **9**: 89-109.

Petersen JS, Rimvall K, Jørgensen PN, Hasselager E, Moody A, Hejnaes K, Clausen JT and Dyrberg T (1998). Regulation of GAD expression in rat pancreatic islets and brain by gamma-vinyl-GABA and glucose. *Diabetologia*, **41**: 530-535.

Pleau JM, Fernandez-Saravia F, Esling A, Homo-Delarche F and Dardenne M (1995). Prevention of autoimmune diabetes in nonobese diabetic female mice by treatment with recombinant acid decarboxylase (GAD 65). *Clin. Immunol. Immunopathol.*, **76**: 90-95.

Roep BO (1996). T-cell responses to autoantigens in IDDM. The search for the Holy Grail. Diabetes, **45**: 1147-1156.

Shirai H, Sato T, Hara T and Minagi S (1998). The effect of diabetes mellitus on histopathological changes in the tissues under denture base and without mechanical pressure. *J. Oral Rehabilitation*, **25**: 715-720.

Shirai M, Yamanaka M, Shiina H, Igawa M, Ogishima T, Fujime M, Ishii N, Okuyama A, Lue TF and Dahiya R (2004). Androgen, estrogen, and progesterone receptor gene regulation during diabetics erectile dysfunction and insulin treatment. *Urology*, **64**: 1244-1249.

Tisch R, Wang B and Serreze DV (1999). Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent. *J. Immunol.*, **163**: 1178-1187.

Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM and Dull TJ *et al.* (1985). Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature,* **313**: 756-761.

Volp ACP, Rezende FAC and Alfenas RCG (2008). Insulin: mechanisms of action and metabolic homeostasis. *Rev. Bras. Nutr. Clin*., **23**: 158-164.

Wang H, Yang J, Jin L, Feng J, Lu Y, Sun Y, Li T, Cao R, Wu J, Fan H and Liu J (2009). Immunotherapy of autoimmune diabetes by nasal administration of tandem glutamic acid decarboxylase 65 peptides. *Immunol. Invest*, **38**: 690-703.

Wei J and Wu JY (2008). Post-translational regulation of L-glutamic acid decarboxylase in the brain. *Neurochem. Res.*, **33**: 1459-1465.

Wherrett DK, Bundy B, Becker DJ, DiMeglio LA, Gitelman SE, Goland R, Gottlieb PA, Greenbaum CJ, Herold KC, Marks JB, Monzavi R, Moran A, Orban T, Palmer JP, Raskin P, Rodriguez H, Schatz D, Wilson DM, Krischer JP, Skyler JS, Type 1 Diabetes Trial Net GAD Study Gr (2011). Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: A randomised double-blind trial. *Lancet*, **378**: 319-327.

White MF and Kahn CR (1994). The insulin signaling system. *J. Biol. Chem.,* **269**: 01-04.

Yashida MH, Da Silva Faria AL and Caldeira EJ (2011). Estrogen and insulin replacement therapy modulates the expression of insulin-like growth factor-I receptors in the salivary glands of diabetic mice. *Anat. Rec*., **294**: 1930-1938.

Yoon JW, Yoon CS, Lim HW, Huang QQ, Kang Y, Pyun KH, Hirasawa K, Sherwin RS and Jun HS (1999). Control of autoimmune diabetes in NOD mice by GAD expression or suppression in beta cells. *Science*, **284**: 1183-1187.

You S, Chen C, Lee WH, Brusko T, Atkinson M, Liu CP (2004). Presence of diabetes-inhibiting glutamic acid decarboxylase-specific, IL-10-dependent, regulatory T cells in native nonobese diabetic mice. *J. Immunol*., **173**: 6777-6785.