Effects of *Candida* on insulin secretion of human adult pancreatic islets and possible onset of diabetes

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ABSTRACT

This study aims to determine the origin of *Candida* contamination of pancreatic tissue cultures, as well as its influence on insulin secretory activity of the pancreatic islets. Pancreatic tissue was obtained after pancreatectomy in patients who had chronic pancreatitis or benign tumours. Islets were isolated under aseptic conditions by a manual method. Microbiological analysis was performed by standard procedures and secretory activity was determined on the first, third and seventh day of cultivation. Insulin stimulation index (SI) on the first day of incubation was 0.665±0.082 and 0.982±0.167 for sterile and infected cultures, respectively (expressed as means±SE). On the third day of cultivation, the SI for sterile cultures was 0.645±0.071 while these value were higher in contaminated cultures (1.252±0.413). On the seventh day, SI was 0.853±0.032 and 1.239±0.169 for sterile and infected cultures, respectively (P=0.05). Analysis of results for the first, third and seventh day of incubation and comparison of both groups showed that SI was 0.721±0.041 for sterile cultures, while for contaminated cultures it was higher by 37.68% (SI =1.157±0.154; P=0.01). The results show that cell culture contamination originates from an original pancreatic tissue infection, and that *Candida* can provoke an elevated level of insulin secretion in such patients, thus increasing chances for the onset of diabetes.

KEY WORDS: Candida.
Insulin.
Diabetes mellitus.
Etiology.

Introduction

Diabetes mellitus (DM) is characterised by metabolic disorder and hyperglycaemia and results from insulin deficiency or decreased effects of insulin on target tissues. The aetiology of DM, and the mechanism of pathogenesis of the disease, is unclear. Type 1 diabetes is characterised by absolute lack of insulin and the physiological destruction of pancreatic islet β-cells. Autoimmune damage to the pancreatic islets is a long-term process and clinical manifestation of the disease occurs at the very end when more than 80% of the β-cells are irreversibly damaged.[1]

Type 2 diabetes is a metabolic disease caused by defective insulin secretion and insulin resistance.[2] It is believed that several factors influence the occurrence of the disease, including genetic factors, reduced physical activity, increased obesity, malnutrition in the fetal and perinatal period, and certain drugs (i.e., steroids, diuretics, antihypertensives). Adipose tissue produces leptin, tumour necrosis factor-α (TNFα), resistin, adiponectin and interleukin (IL)-6, and thereby affects insulin resistance and possible dysfunction of pancreatic β-cells.[3–5]

One of the important factors that might affect insulin secretion is an infection of the pancreas. Bacterial infection of pancreatic islet cultures may reduce or increase functional capacity (i.e., insulin secretion as a response to in vitro stimulation with high and low glucose concentrations) depending on the bacterial species. On the other hand, pancreatic infection with those bacteria can provoke reduced or elevated level of insulin secretion in such patients, thus increasing the chance of the onset of diabetes.[6] Exposure to multiple pathogens could cause a chronic low-grade inflammation, resulting in insulin resistance.[7] But how does an infection of the pancreas occur and what are the effects and types of infection.

In patients with acute pancreatitis the inflammation could get worse when infection is present, leading to the expansion of inflammation and necrosis, and typically occurs after the bsecond or third week of serious illness. It may be associated with a wide range of Gram-positive and Gram-negative bacteria, especially *Enterobacter* and *Candida* spp.[8]

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Severe cases of acute pancreatitis might lead to numerous complications. A damaged pancreas could be infected by bacteria from the small intestine. Symptoms of inflammation include fever, increased number of leucocytes and, in severe cases, organ failure.

In patients with necrotic pancreatitis, infection is caused by *Enterobacter* in 58% of cases, while in 42% it was caused by infected venous catheter, urinary tract, tracheal mucosa or biliary system infections. In addition to Gram-positive and Gram-negative bacteria and multiresistant organisms, in 24% cases fungi were isolated.[9]

Pseudocyst pancreatic infection by *Candida albicans* can cause sepsis or death due to multiple organ failure.[10] Intra-abdominal infections by *Candida* caused by spontaneous perforation or surgical opening of the gastrointestinal tract may increase the overall mortality of patients.[11] Infection of the pancreas originates in the duodenum and some studies have identified infections of pancreatic duct (ductus pancreaticus) with *Candida*.[12,13]

C. albicans is a commensal and a constituent of the normal

microflora in 80% of the human population, and predominately colonises the mucosal surface of the gastrointestinal tract, genitourinary tract and, to a lesser extent, the skin.[14,15] However, especially in immuno-compromised patients (e.g., cancer chemotherapy, AIDS, organ transplantation or in neonates) or when the competing flora are eliminated (e.g., after antibiotic treatment), *C. albicans* becomes an opportunistic pathogen that can cause superficial but also systemic and potentially life-threatening infections.

It is a general view that the pathogenicity of *Candida* spp. is not caused by a single dominant virulent strain.[16,17] Virulence is possible only when all three vegetative and morphological forms (i.e., yeast, pseudohyphae and hyphae) are expressed.[18] It seems that *C. albicans* has high adaptability on different host niches as illustrated by the possession of different adhesins that mediate binding to various tissues, as well as elongated hyphae that can avoid or escape the phagocyte cells and yeast-like cells that can spread through the bloodstream, thus allowing the fungus colonisation and infection of almost all body locations.[19]

The aim of the present study is to investigate *C. albicans* infection of pancreatic islet culture, as well as the influence of infection on insulin secretion.

Materials and methods

Human adult pancreatic tissue was procured from the Institute for Gastrointestinal Diseases, Clinical Center of Serbia. Tissue samples were collected from live donors, after total or subtotal pancreatectomy for cysts or tumours.[20] In tumours, healthy tissue was obtained near the line of the resection. Histopathological analysis of tissue samples showed no tumour cells. All procedures were performed in accordance with the rules of the Ethical Committee of the Medical Faculty in Belgrade. Written consent was obtained from all patients.

Pancreatic tissue was transported in physiological solution (0.9% sodium chloride) in sterile vessels (75 mL volume) from the Institute for Gastrointestinal Diseases to the Laboratory for Pancreatic Islets Culture in the Institute for Endocrinology, Diabetes and Metabolic Diseases. The material was kept in the refrigerator on 4°C (cold ischaemia). Warm ischemia is the time measured from the beginning of the isolation procedure to the moment when the islets were placed in culture medium. For this study, islets from 13 pancreata were employed, seven were infected by *C. albicans* and six were sterile. In the infected group, cold ischaemia was 80.43±14.07 min and warm ischemia was 112.43±15.5 min. Average weight of pancreatic tissue was 4.54±1.05 g. In the non-infected group, cold ischaemia was 55.67±5.69 min and warm ischemia was 129.5±10.5 min. Average weight of pancreatic tissue was 4.1±1.06 g (Table 1). To establish that microorganisms originated from the pancreas, three microbiological analyses were performed: i) immediately near the line of the resection of the pancreas; ii) from the pancreatic tissue after its transport to the laboratory and prior to isolation; and iii) at the end of the experimental procedure (to eliminate the possibility of contamination during the incubation).

Microbiological analyses were performed by standard procedures at the Institute for Microbiology, Clinical Center, Serbia.

Table 1. Biometric data of samples.

	Tissue contaminated with *Candida albicans*	Sterile pancreatic samples
Cold ischaemia (min)	80.43±14.07	55.67±5.69
Warm ischaemia (min)	112.43±15.5	129.5±10.5
Average weight of pancreatic tissue (g)	4.54±1.05	4.1±1.06

Isolation of islets

Isolation of the adult pancreatic islets was performed in aseptic conditions in a laminar cabinet. Tissue was transferred to Hanks solution (Sigma-Aldrich) and mechanically chopped. This material was collected with a pipette and put in a test tube containing liberase (Roche Diagnostics; concentration 1.5 mg/mL,[21] and was prepared by dissolving enzyme in distilled water maintained in a refrigerator for 30 min prior to use. Working concentration was adjusted by adding the Hanks solution. Liberase solutions were sterilised using a cellulose acetate membrane filter (0.22 µm porosity). Duration of incubation was 30 min at 37°C with occasional mechanical stirring. After incubation, the content of the test tube was centrifuged at 400 x*g*, for 10 min at 15°C. Supernatant was decanted and the remaining islets were rinsed several times with Hanks solution to remove excess lipid and liberase. Islet yield was 2–4000 IEQ/g pancreatic tissue.

After rinsing, the islets were resuspended in the final RPMI medium 1640 (Sigma-Aldrich) supplemented with 0.1% L-glutamine, 5.5 mmol glucose, 25 mmol/L Hepes, 100 units/mL penicillin, 100 µg/mL streptomycin and 10% fetal calf serum (FCS, Sigma). The islets were incubated in plastic flasks (Falcon; 50 ml volume) in an incubator at 37°C in a 5% CO_2 and 95% humidity atmosphere for seven days.

Determination of the number and viability of isolated islets

Viability of the islets was determined by dithizone (DTZ) staining on days 1, 3 and 7 after isolation. Viability was expressed as percentage of distinctly stained (red) islets in relation to total number of the islets in culture.

Preparation of the dithizone solution

Dithizone (50 mg) was dissolved in 10 mL dimethyl sulphoxide (DMSO) and 10 ml Hanks solution. The solution was filtered through a nylon filter (0.20 µm). Samples (1 mL each culture) were stained with 0.2 mL DTZ solution and incubated for 30 min. Stained islets were rinsed in Hanks solution and resuspended in 1 mL RPMI medium. The number of the cells was determined using a stereomicroscope and special micro-chambers.[22]

Determination of functional capacity and insulin secretion

To determine preservation of the functional capacity of the isolated islets, glucose-stimulated insulin secretion was measured on the first, third and seventh day of cultivation. A static glucose stimulation assay was also performed.

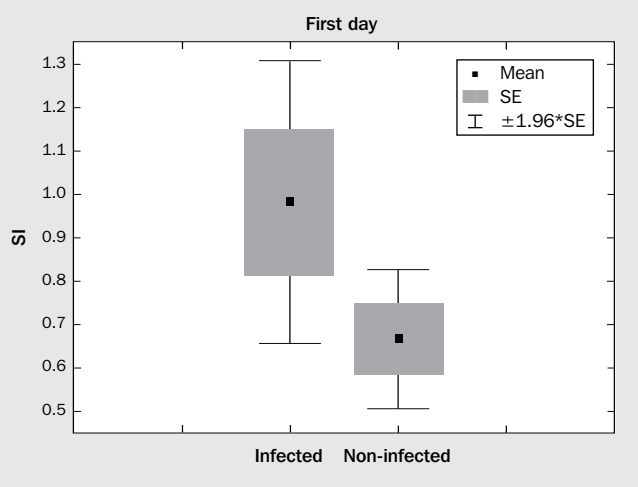

Fig. 1. Insulin secretion (SI) in response to low and high glucose stimulation in sterile cultures (control group) and cultures infected with *Candida albicans* on the first day of incubation. Stimulation index (mean±SE) value in sterile cultures was 0.665±0.082 and in infected cultures was 0.982±0.167 ($P>0.05$).

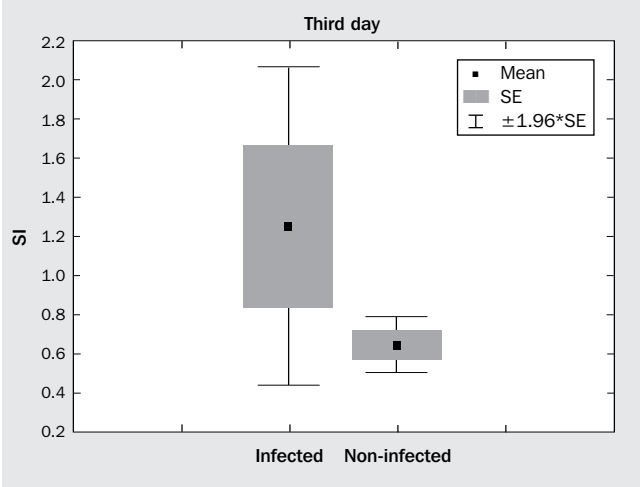

Fig. 2. Insulin secretion (SI) in response to low and high glucose stimulation in sterile cultures and cultures infected with *Candida albicans* on the third day of incubation. Stimulation index (mean±SE) value in sterile cultures was 0.645±0.071 and in infected cultures was 1.252±0.413 ($P>0.05$).

Samples were incubated for 1 h in low glucose (2.8 mmol/L RPMI) medium, than 1 h in high glucose (20 mmol/L RPMI) medium and 1h in low glucose medium again. After each step of stimulation, cultures were centrifuged at 400 x*g* for 10 min at 15°C. Supernatant was decanted and stored at –18°C for insulin quantification. Insulin content was determined by radioimmunoassay. Sensitivity of the assay was 0.60 miu/L and the detection range was 0.6–300 miu/L. Relative insulin release was expressed as a stimulation index (SI) and calculated as the ratio of insulin release during high glucose stimulation to insulin release during low glucose stimulation. The number of stimulated islets do not affect the SI value.

Statistical analysis

Statistical analysis was performed using Student's *t*-test for independent samples. Statistica for Windows 6 was used to evaluate the differences in means between two groups (variables). $P\leq0.05$ was regarded as statistically significant. Analysis of infected and non-infected cultures was performed on the first, third and seventh days of infection, and for entire period of infection, respectively.

Results

In total, 29 samples of human pancreatic tissue were analysed. Nine samples (31%) were contaminated by bacteria (*Pseudomonas aeruginosa* [16.6%], *Enterobacter* spp. [33.3%] and *Staphylococcus* spp. [11.1%]), seven (24%) were contaminated with *C. albicans*, and 13 samples were sterile (control group).

Comparison of SI values for the two examined group, sterile cultures and those infected with *C. albicans* showed that the difference between two groups on the first day of incubation was not statistically significant ($P>0.05$). In sterile cultures, insulin SI was 0.665±0.082 (means±SE) and in contaminated cultures was 0.982±0.167 (Fig. 1). Insulin secretion was higher by 31.3% in infected cultures. On the third day of incubation, SI in sterile cultures was 0.645±0.071 and in infected cultures was higher by 48.5% (SI = 1.252±0.413; Fig. 2). The difference was not statistically significant ($P=0.199$). On the seventh day, in sterile cultures the SI was 0.853±0.032, while in cultures infected with *Candida* the SI was higher by 31.15% (SI = 1.239±0.169; Fig. 3). The difference was statistically significant ($P=0.05$). The analysis of results for the first, third and seventh day of incubation and for both groups showed that in sterile cultures SI was 0.721±0.041 and in cultures infected with *Candida* the SI was 37.68% higher (SI = 1.157±0.154). This difference was statistically significant ($P=0.01$; Fig. 4).

Discussion

The genus *Candida* includes more than 350 species present in humans, other mammals, birds, fish, insects, arthropods, animal waste, plants and substrates naturally rich in sugars (e.g., honey, nectar, grapes, fermentation products and dairy products), fresh water, seawater and particles in the air.[23–25]

At least 13 *Candida* species cause infection in humans. The most common of these are *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*. High incidence of *Candida* infection is represented by the fact that nearly three-quarters of all healthy women experience at least one vaginal yeast infection.[26] As a result, Candida has become the fourth most common nosocomial bloodstream pathogen in the USA.[27]

The effects of *Candida* spp. on the human body is not well understood, and therefore this study aimed to examine the influence of *Candida* infection in pancreatic islets cultures on insulin secretion *in vitro* in short-term (seven day) incubation. Analysis of the results (Figs. 1–3) shows that cultures infected by *Candida* have higher insulin secretion in response to glucose stimulation than the sterile one. On the first day of cultivation, insulin secretion was higher by 31.3%, the difference increased to 48.5% on the third day and than decreased to 31.15% on the seventh day.

During cultivation, SI values of infected cultures increased on the third and seventh day compared to the first day (0.982, 1.252 and 1.239 on the first, third and seventh day,

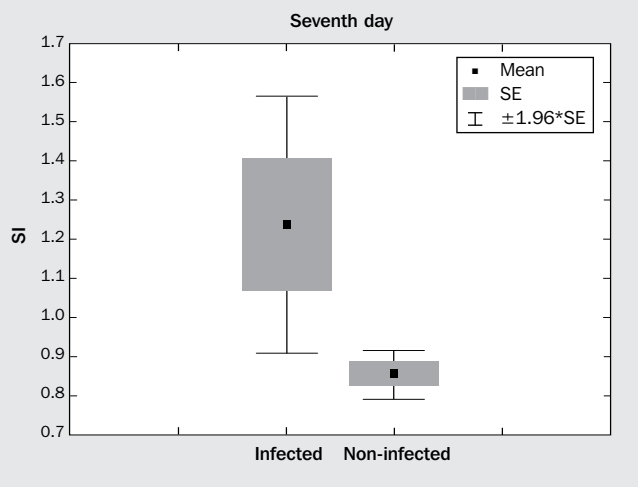

Fig. 3. Insulin secretion (SI) in response to low and high glucose stimulation in sterile cultures and cultures infected with *Candida albicans* on the seveth day of incubation. Stimulation index (mean±SE) value in sterile cultures was 0.853±0.032 and in infected cultures was 1.239±0.169 (P=0.05).

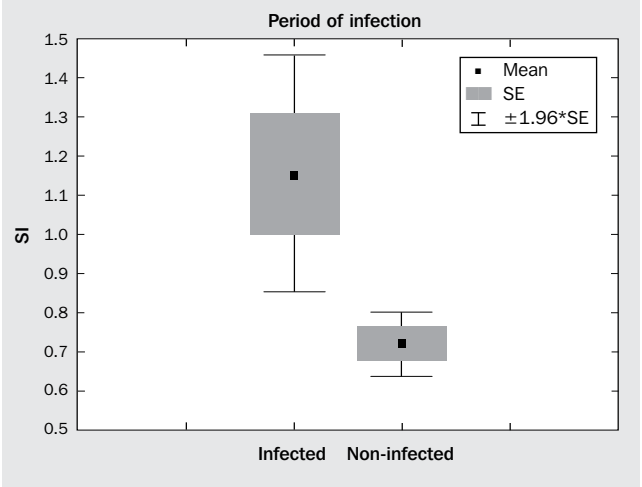

Fig. 4. Insulin secretion (SI) in response to low and high glucose stimulation in sterile and infected cultures (*C. albicans*): summary of results for the first, third and seventh day of cultivation. Stimulation index (mean±SE) in sterile cultures was 0.721±0.041 and in infected cultures was 1.157±0.154 (P=0.01).

respectively). Analysis of the sumary results for both groups on the first, third and seventh day shows that *Candida* increases insulin secretion of pancreatic islets in culture. In sterile cultures, SI value was 0.721±0.041 , while in infected culture the SI was 1.157±0.154 and the difference was statistically significant (P=0.01; Fig. 4). But how does *Candida* exert this effect and causes an increase in insulin secretion in infected cultures.

When cultured in standard yeast laboratory medium (low temperatures and pH) *Candida* grows mostly as ellipsoid yeast cells. However, mild changes in temperature and pH can result in morphological shift to pseudohyphal growth. At temperature of 37°C and neutral pH and in response to external stimuli (e.g., serum), long, narrow hyphae develop from yeast cells. At intermediate temperatures and pH, elongated pseudohyphal cells develop. Hyphae rarely produce pseudohyphal buds and pseudohyphae rarely form true hyphae.[28] Pseudohyphal cultures always contain some yeast and/or some hyphal cells.

In mixed cultures there are interactions between pancreatic islet cells and *Candida* cells. Interaction of *Candida* with other cells is highly specific. Incubation of *Candida* cells and fibroblasts for 3 h at 37°C leads to a close bond between budding yeast cells and the fibroblasts, independently of glucose concentration in the medium. However, a similar reaction is not observed with renal epithelial cells.

C. albicans is a highly adaptable species to different environmental conditions. No single factor accounts for *Candida* virulence, but it has developed a variety of mechanism that allow successful colonisation/infection and evasion of host responses. These factors include hyphal formation, surface recognition molecules (adhesins), phenotypic switching and extracellular hydrolytic enzymes.

High adaptability of *Candida* is evident in the possession of the *SAP* gene family, with 10 members encoding secreted aspartyl proteinases.[29] These enzymes are linked with the virulence of the fungus.[30] Proposed functions of the proteinases during infection include the digestion of host proteins for nutrient supply, the evasion of host defences by degrading immunoglobulins and complement proteins, and adherence and degradation of host barriers during invasion.[31]

In the present study, *Candida* did not act as a parasitic form but as a typical commensal. Even when proteinases damage cells and hence reduce their number, increased insulin secretion cannot be explained, as the number of stimulated cells did not affect SI values. It is possible that there is activation of certain specific genes of SAP family, as they are differentially regulated and they are expressed under various laboratory growth conditions and during experimental *Candida* infections *in vitro* and *in vivo*.

The role of *SAP* genes in *Candida* pathogenesis has been demonstrated using *SAP*-deficient mutants and proteinase inhibitors. Different *SAP* genes are of great importance for mucosal and systemic infections. It has been demonstrated that they are involved in *C. albicans* adherence, tissue damage and evasion of host immune responses.[30]

Candida infection originated from infected pancreatic tissue and it can be isolated from the pancreatic duct, indicating that infection can be transfer from the duodenum to the pancreas.[12,13] In an animal model (mice), parenchymal organs such as the liver and pancreas are invaded by *C. albicans* wild-type hyphal cells between 4 h and 24 h after intraperitoneal infection.

One of the factors contributing to *Candida* virulence is the ability to change between yeast and hyphal cells (dimorphism). The pathogenesis of candidosis involves adhesion and penetration of hyphal cells from a colonised mucosal site to internal organs. Other virulence factors include adhesion factors, thigmotropism, phenothypic switching and other hydrolitic enzymes.[32–35]

Candida yeasts are commonly present in humans but their growth is normally limited by the immune system and by other microorganisms occupying the same locations of the body.

Patients with weakened immune status/immuno-compromised patients (e.g., HIV-infected patients) suffer persistent *Candida* infections.[15] The use of antibacterial therapy can increase incidence of yeast infections as well as diabetes mellitus.[11,36]

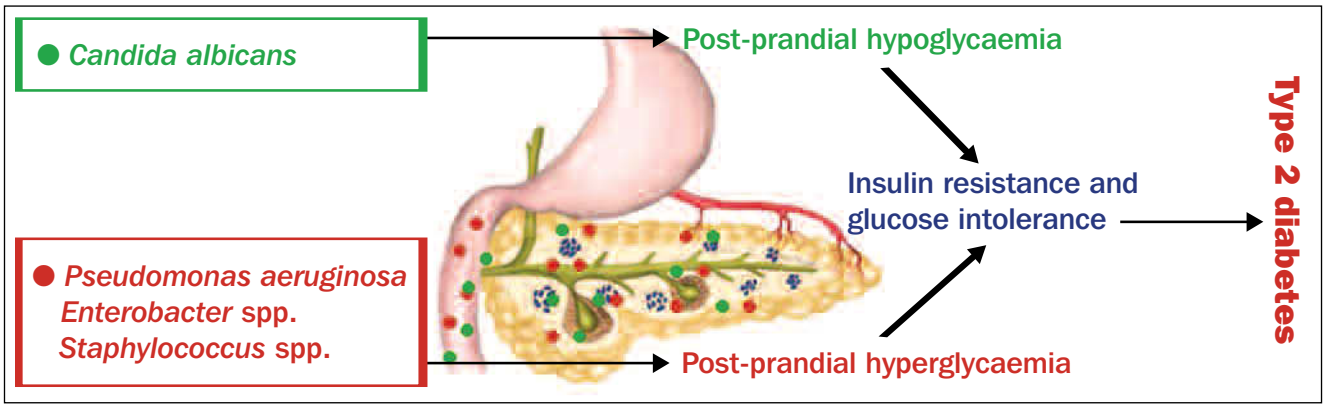

Fig. 5. *Candida* and bacterial influence on the development of type 2 diabetes.

Immune response to *Candida* infection in human is not well known. It is not likely that yeast growth correlates directly with activation of gut-associated lymphoid tissue (GALT). Saprophyte bacteria that normally live in the nintestine have a substantial influence and antibacterial therapies in diabetics patients may disrupt this relationship.

Studies on mice have shown that resistance to oral and gastrointestinal infections depends on CD4-positive T cells.[37] Another important factor in immune response to infections are IL-12, TNFα and phagocytes.[38,39] It also has been shown that the complement component C5 limits the initial yeast growth in the kidney.[40] Research on immunodeficient and T-cell-depleted mice suggests that clearance of the yeast may predominantly be a function of the innate response, whereas the adaptive response may either limit tissue damage or have the potential to cause immunopathology, depending on the host genetic context in which the infection takes place.[41,42]

Based on the results, it can be assumed *Candida* infection of the pancreas may lead to destruction of pancreatic β-cells due to activation of the immune system as a response to existing infection, and thereby induce the onset of diabetes type 1.

In patients with type 2 diabetes, in addition to insulin resistance there is diminished insulin secretion in response to glucose stimulation (post-prandial hyperglycaemia). Degradation of glycogen in the liver leads to a glycotoxic state which increases β-cell apoptosis. On the other hand, increased insulin secretion has negative effects on physiological functions of β-cells. Insulin resistance involves a decline in insulin action within the body, which eventually results in the loss of insulin secretion, the onset of glucose intolerance, and, in some cases, the development of diabetes.[43] There is also the likelihood of an elevated CRP concentration with increasing HbA1c levels.[44] These findings suggest an association between glycaemic control and systemic inflammation in people with established diabetes. Exposure to multiple pathogens could cause chronic low-grade inflammation, resulting in insulin resistance.[7]

Conclusions

If present in the pancreas, *Candida* in interaction with pancreatic islet cells may cause increase insulin secretion *in vivo*. It is known that most diabetic patients have persistent mucous *Candida* infections. In addition, *Candida* as a commensal in the digestive tract can secrete its products and reach the pancreas via the bloodstream, thus affecting insulin secretion indirectly, especially in patients after long-term antibiotic therapy,[11,15] which offers direct proof that intestinal saprophytes (bacteria) normally keep *Candida* growth under control.

Microorganisms from the intestine can enter the pancreas in three ways, via the blood, penetration into body cavity due to injury, and directly from the duodenum through the pancreatic duct (ductus pancreaticus).

The results presented in this study suggest that *Candida* infection of pancreatic cultures may increase functional capacity (i.e., insulin secretion as a response to *in vitro* stimulation with high and low glucose concentrations). In islet culture, *Candida* is a typical commensal. Although proteinases damage the cells, reducing their number, increased insulin secretion cannot be explained because the number of stimulated cells does not affect SI values.

Results of *in vitro* research demonstrate that microorganisms can influence pancreatic islets insulin secretion directly (when present in the pancreas) and indirectly (increased in number in gastrointestinal tract),[45] bacterial infections (*Enterobacter* spp, *Pseudomonas aeruginosa*, *Staphyloccocus* spp) reduce insulin secretion, leading to post-prandial hyperglycaemia,[6] and fungal infections (*C. albicans*) increase insulin secretion, causing post-prandial hypoglycemia and insulin resistance. Both cases lead to glucose intolerance and insulin resistance, and in some cases the development of type 2 diabetes (Fig. 5).

Based on the results presented, it can be concluded that Candida infection of the pancreas and changes in saprophytic flora are important factors involved in the pathogenesis of diabetes. □

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