

A comparison of the severity of oral candidiasis between gestational and type 1 diabetes mellitus

Ayu Ragil Destrian Pangestu,¹ Siti Nosya Rachmawati,¹ Leni Rokhma Dewi,² and Mei Syafrjadi¹

¹Laboratorium of Oral Pathology, Department of Biomedical Sciences

²Department of Oral Medicine

Faculty of Dentistry, Universitas Jember, Jember – Indonesia

ABSTRACT

Background: Diabetes mellitus is a metabolic disorder caused by insufficient insulin production due to pancreatic β cell destruction, whereas in gestational diabetics an increase of hormone estrogen induces insulin resistance. Oral candidiasis constitutes an opportunistic fungal infection due to a compromised immune system that is a medical condition reported by diabetics, including those suffering from gestational diabetes. **Purpose:** The study aimed to determine the severity of oral candidiasis in female Wistar rats with type 1 and gestational diabetes mellitus. **Methods:** This research constituted a laboratory experiment incorporating a post test-only group control design whose subjects were female Wistar rats divided along the following lines: group 1 consisted of diabetic non-pregnant rats, group 2 contained diabetic pregnant rats induced by streptozotocin and the control group members constituted normal female rats. Diabetes induction was performed by means of 40 mg/kgBW streptozotocin administrated intraperitoneally. Diabetes mellitus was confirmed when the blood glucose level ≥ 120 mg/dL. All groups were exposed to 0.2 ml *Candida albicans* (*C. albicans*) suspension (5×10^8 CFU/ml) in the oral buccal vestibule between the distal incisors and mesial maxillary first molar for three days. A swab was performed on the third day after final exposure before the samples were observed under a light microscope. *C. albicans* cultivation and calculation of the resulting colonies was carried out on Sabouraud dextrose agar after they had been identified by means of a germ tube test. **Results:** The result confirmed the absence of hyphae in the control group, while in group 1 all samples contained hyphae. Moreover, group 2 featured a dense hyphae population. A chi-square test indicated a statistical significance ($p < 0.05$) between all groups. **Conclusion:** Oral candidiasis in gestational diabetes is more severe than that occurring during type 1 diabetes mellitus.

Keywords: candida; diabetes type 1; gestational

Correspondence: Mei Syafrjadi, Laboratorium of Oral Pathology, Department of Biomedical Sciences, Faculty of Dentistry, Universitas Jember, Jember – Indonesia. Email: didiriadihsb@gmail.com

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder with characteristics of hyperglycemia that occurs due to abnormal insulin secretion or the inability of the body to process insulin effectively. Insulin is a hormone that regulates blood sugar levels the deficiency of which causes an increase in the concentration of glucose in the blood (hyperglycemia).^{1,2} Gestational diabetes results from increased secretion of the hormone estrogen which has a metabolic effect on glucose tolerance, while pregnancy is a diabetogenic state. This hormonal factor results in insulin resistance and, ultimately, hyperglycemia.¹

Oral candidiasis constitutes one of the opportunistic fungal infections of the oral mucosa caused by *Candida albicans* (*C. albicans*) whose symptoms include white patches that are confluent and adhere to the oral mucosa and pharynx, particularly affecting the mouth and tongue.^{3,4} The most prevalent form of lesion presented by sufferers of diabetes mellitus is white plaque of a pseudomembranous type (oral thrush) most commonly found on the dorsum of the tongue.^{5,6}

Diabetes mellitus is a predisposing factor in the onset of oral candidiasis especially in pregnant patients since hormonal changes occurring in the body of an expectant woman render her more susceptible to *C. albicans* infection.

In particular, elevated levels of the hormone estrogen cause high levels of glycogen, thereby providing a sufficient source of carbon to support the growth of *C. albicans*.⁷

Several studies have investigated the proportion of individuals within a specific population suffering from diabetes mellitus with oral candidiasis without establishing its severity in terms of the number of *C. albicans* spore colonies in diabetics who present candidiasis. Contrastingly, in the field of gestational diabetics, a number of studies have only examined *C. albicans* infection in the vaginal region, while scant research has been conducted on the oral cavity.

Based on the background outlined above, an investigation of oral candidiasis infections under diabetic conditions compared the relative severity of diabetes mellitus and gestational diabetes mellitus infections by observing the growth of spora and hyphae after exposure to oral mucousal containing *C. albicans*. It is hoped that this study will demonstrate how the maintenance of stable blood sugar levels can provide protection against oral candidiasis infection, especially in diabetes mellitus patients.

MATERIALS AND METHODS

The research constituted a laboratory experiment with a post test-only group control design involving a total of 12 female Wistar rats (*Ratus norvegicus*) divided into three groups of four samples in accordance with Notoatmodjo's theory.⁸ Ethical approval of the research was granted through Ethic Committee Approval Number 108/UN25.8/KEPK/DL/2018, issued by the Faculty of Dentistry, University of Jember.

The sample group consisted of healthy, fully mobile, pregnant and non-pregnant female *Rattus norvegicus*, weighing 150-300 grams which were free of eye disease. The control group contained only healthy rats, while the samples in treatment group 1 consisted of diabetic non-pregnant rats and treatment group 2 were diabetic pregnant rats. The diabetes mellitus samples in treatment groups 1 and 2 were induced by *streptozotocin* (STZ) at a dose of 40 mg/kg BW dissolved in 50 mg / ml 0.1 M *citric acid buffer* (pH 4.5) administered intraperitoneally.^{9,10} Those samples with blood glucose levels \geq 120 mg/dL were categorized as suffering from diabetes mellitus.¹⁰ All sample groups were exposed to 0.2 ml *C. albicans* suspension (5×10^8 CFU/ml) derived from the oral buccal vestibule between the distal incisors and the mesial maxillary 1 molar for three days.¹¹ A swab was taken on the third day after final exposure. The *C. albicans* used during this research was derived from the candidal culture held by the Microbiology Laboratory at the Faculty of Dentistry, Jember University.

A swab procedure was performed on the buccal vestibule using a plastic filling instrument until the white plaque had been removed and the base of the lesion appeared reddish in colour. The resulting swabs were smeared on a glass slide before being observed under a light microscope

(Olympus CX 21 LED) in order to view any hyphae and spores, while also establishing the density of the hyphae population. Furthermore, the candidal on the glass slide was added to 0.5 ml of sterile aquadest and mixed to form candidal suspension.¹² A total of 0.1 ml of *C. albicans* suspension was removed from the glass slide by means of a pipette and applied to *Sabouraud dextrose agar* (SDA) in order to culture *C. albicans* spore colonies for 24-48 hours at 37°C.¹² The respective sizes of the resulting *C. albicans* colonies present on the media were then calculated using a colony counter.¹³

Calculation of spore colonies on the SDA media was undertaken three times by different observers. Spore colonies grown in a petridish were placed on the colony counter and divided into four quadrants two of which contained seven boxes while the other two quadrants contained eight boxes, giving a total of 30 boxes. The results provided by the three observers were averaged and entered into the formula $CFU/ml = \text{number of colonies} \times 1/\text{dilution}$. For the purposes of this research, dilution was set at 10^{-3} .^{12,14}

The identification of *C. albicans* involved the use of 2 ml of chicken egg white as the culturing medium which was incubated for 30 minutes at 37°C. *C. albicans* suspension from the swab was added and then incubated for 2-3 hours at 37°C. Positive *C. albicans* was found in the form of cells that germinate in the form of a germ tube when observed under a light microscopic.¹⁵ Histopathological examination was performed from oral mucosa that was stained with Hematoxylin Eosin (HE).

Parametric qualitative data relating to the severity of oral candidiasis and morphology of *C. albicans* was analyzed descriptively. Statistical analysis of the data was undertaken through the conducting of a chi-square test. If the p-value < 0.05 , this indicated the existence of a significant relationship between the rows and columns.

RESULTS

Examination of candidal spores obtained by means of oral mucosal swab confirmed that all control group samples (100%) contained spores, but no hyphae growth occurred. In contrast, treatment group 1 contained spores and hyphae (100%), while in the treatment group 2 spores were found in all samples (100%) but hyphae growth only occurred in 75% of them (Table 1). A *chi-square* test confirmed the significance (p-value) to be 0.012 ($p < 0.05$), indicating that there was a significant correlation between all groups due to the presence of candidal hyphae.

Hyphae quantity scoring is based on both the presence and density of hyphae and can be undertaken to distinguish their severity. In the control group, there were no (negative/-) hyphae, while in treatment group 1 the hyphae scoring was positive (+1) (not dense population) leading to a classification of mild oral candidiasis. In treatment group 2, 50% of the samples contained a dense hyphae

Table 1. Presence of candidal spores and hyphae in oral mucosal swabs

Group	Sample	Spore	Hyphae
Control group (Normal subjects)	1	Yes	No
	2	Yes	No
	3	Yes	No
	4	Yes	No
Treatment group 1 (Type 1 diabetes mellitus subjects)	1	Yes	Yes
	2	Yes	Yes
	3	Yes	Yes
	4	Yes	Yes
Treatment group 2 (Gestational diabetes subjects)	1	Yes	Yes
	2	Yes	No
	3	Yes	Yes
	4	Yes	Yes

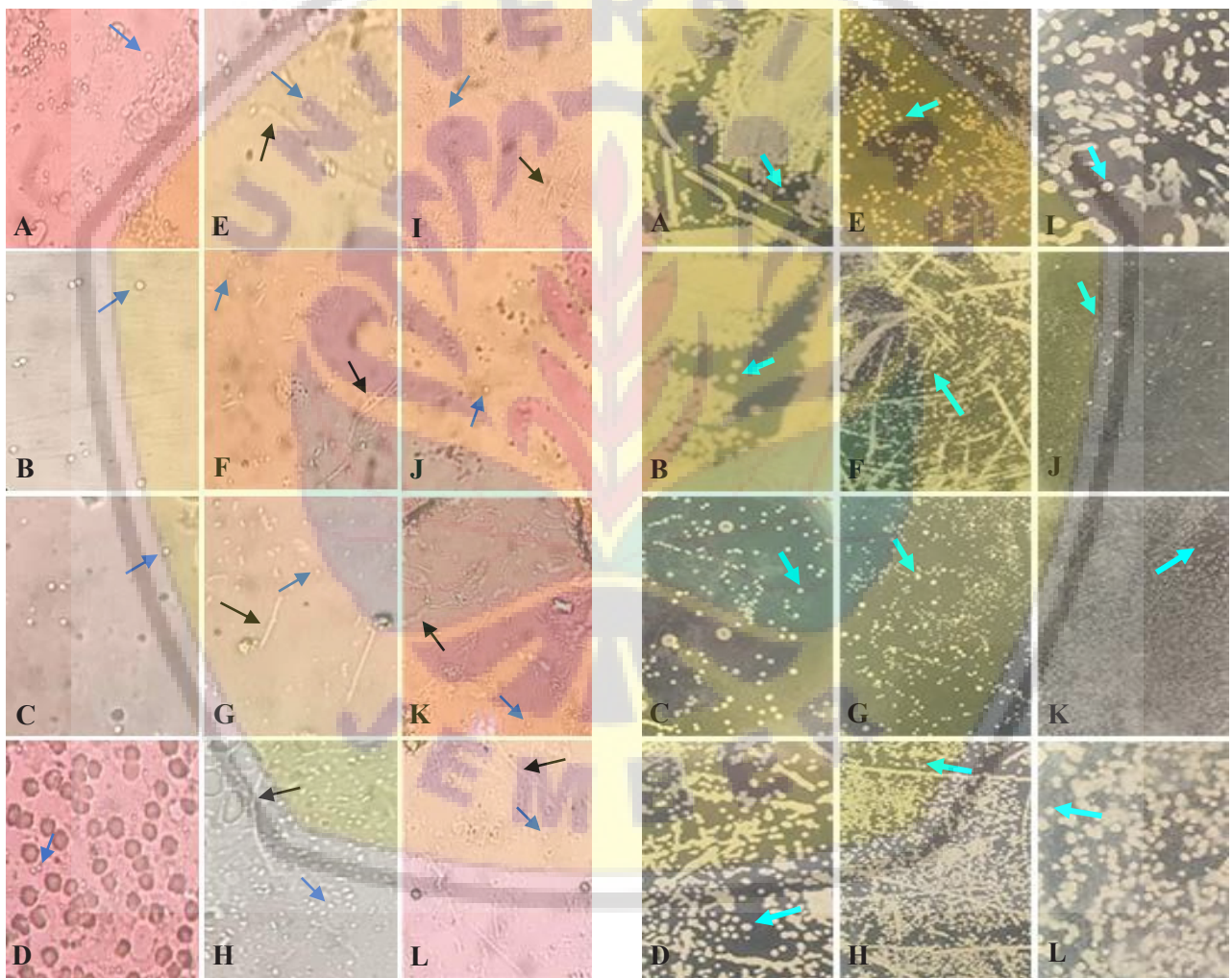


Figure 1. Microbiological pictures of spores (blue arrow) and hyphae (black arrow) obtained from an oral mucosal swab. Control group (A-D), Treatment group 1 (E-H), Treatment group 2 (I-L). 400x magnification.

Figure 2. Control group (A-D), Treatment group 1 (E-H), Treatment group 2 (I-L). Pictures of *Candida albicans* spore colonies (blue arrow) on SDA culture media. Spore colonies are identified as *Candida albicans* if they are circular with a slightly convex surface, smooth, slippery and yellowish white with a sour aroma similar to that of yeast.

population (+2) leading to a classification of severe oral candidiasis, one sample indicated sporadic hyphae growth (+1), while in another sample no candidal hyphae were located (Figure 1 and Table 2). Statistical tests produced a p-value of 0.006 ($p < 0.05$), confirming a significant correlation between all groups due to hyphae growth or the quantity of hyphae.

The calculation of spore colonies on the SDA media in all groups was >400 CFU/ml, which meant that 100% of the subjects were at risk of developing oral candidiasis (Table 3). The term TNTC (Too Numerous To Count) signifies a situation in which the number of colonies on

the media is too high to be calculated. This can occur because of low dilution factor levels resulting in a high concentration or uneven distribution of *C. albicans* in the suspension, in turn causing an accumulation of *C. albicans* spore colonies which renders calculating them difficult.¹⁶ The calculations of the spore colonies present in the media can be seen in Figure 2 and Table 3.

Histopathological images confirmed the presence of mucosal epithelial hyperplasia and dense inflammatory cells in the lamina propria indicating a host cell immune response to the presence of *C. albicans* which resulted in inflammation (Figure 3).

Table 2. The hyphae quantity scores

Group	Sample	Hyphae
Control group (Normal subjects)	1	(-)
	2	(-)
	3	(-)
	4	(-)
Treatment group 1 (Type 1 diabetes mellitus subjects)	1	(+1)
	2	(+1)
	3	(+1)
	4	(+1)
Treatment group 2 (Gestational diabetes subjects)	1	(+2)
	2	(-)
	3	(+2)
	4	(+1)

(-): No hyphae;
 (+1): Sporadic hyphae growth (mild candidiasis);
 (+2): Dense hyphae (severe oral candidiasis)

Table 3. Calculation of spore colonies in SDA media

Group	Sample	Number of spore colonies (CFU/ml)
Control group (Normal subjects)	1	TNTC
	2	TNTC
	3	2×10^5
	4	1.9×10^5
Treatment group 1 (Type 1 diabetes mellitus subjects)	1	2.5×10^5
	2	4.2×10^5
	3	4.2×10^5
	4	4.1×10^5
Treatment group 2 (Gestational diabetes subjects)	1	6.6×10^5
	2	TNTC
	3	TNTC
	4	5.1×10^5

*TNTC: Too Numerous To Count
 Normal: < 400 CFU/ml
 Risk of oral candidiasis: >400 CFU/ml

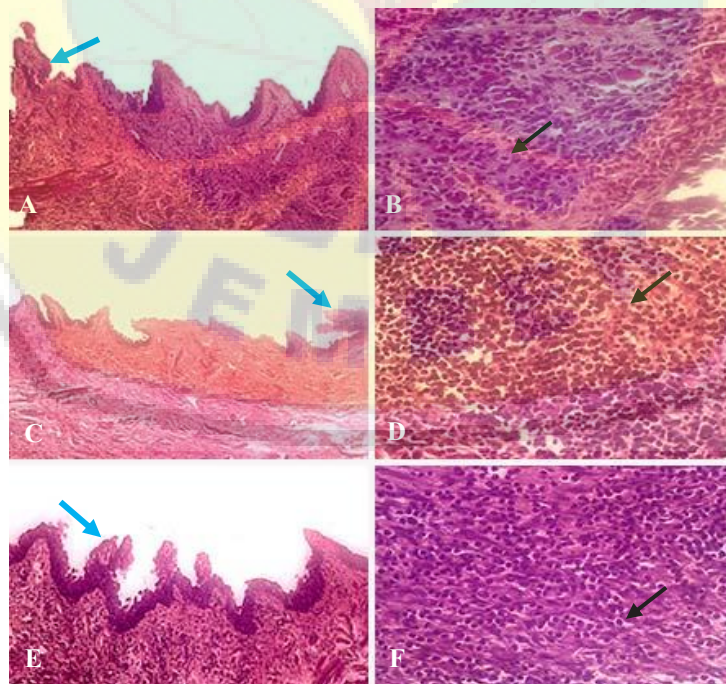


Figure 3. Histopathological appearance showed focal hyperplastic epithelium of oral mucousal (blue arrow) and dense inflammatory population in lamina propria (black arrow) due to the presence of spora >400 CFU/ml and hyphae. Control group (A-B), Treatment group 1 (C-D), Treatment group 2 (E-F). (HE staining, A-E: 100x and B-F: 400x magnification).

DISCUSSION

Candida albicans constitutes a normal flora found in the human oral cavity in the form of spores. Under certain conditions, for example diabetes, the growth of the *C. albicans* becomes excessive resulting in infection,¹⁷ as was the case in this study where all groups were infected after oral mucosal exposure to multiplying *C. albicans* spores. The research findings related to treatment groups 1 and 2, the members of which all suffered from diabetes mellitus whose spores may develop into hyphae. Increasing the number of spores and degree of hyphae penetration can potentially produce an oral mucosal infection. Histopathological observation confirmed dense inflammation infiltration and focal epithelial hyperplastic as a host immune response to lamina propria. Given the nature of this infection, all samples could be classified as mild to severe cases of oral candidiasis. The severity of oral candidiasis was found to be in gestational diabetes mellitus compared to type 1 diabetes mellitus.

The contents of Table 1 indicate the *C. albicans* growth on the buccal mucosal surface of all samples after exposure for three days. Observation confirmed both their spore form and hyphae-like fungal form. *C. albicans* can be easily detected because it is an organism that has two forms (dimorphic organisms), namely; spores that form a yeast-like substance (non-invasive) and hyphae-like fungal forms that produce root-like rhizoid structures capable of penetrating the mucosa (invasive) and causing infection (oral candidiasis).⁴ In this study, it was found that hyphae-like fungal forms invasive to oral mucosal tissue produced an infection which appeared histopathologically as an immune response such as dense inflammatory cells in the subepithelium (Figure 3.). This infection affected both treatment groups 1 and 2 who suffered from diabetes.

The contents of Table 2 show that no hyphae were found in any samples contained in the control group. Only spores numbering more than 400 CFU/ml were present. These were capable of producing an infection in the buccal mucosa which, histopathologically, appeared as dense inflammatory cell infiltration in the lamina propria (see Figures 3A and 3B). Meanwhile in treatment group 1 (type 1 diabetes mellitus group), all samples were found sporadically in both the spores and candidal hyphae (Score +1). Therefore, based on the scoring system utilised, they were classified as mild oral candidiasis and this condition also presents inflammatory cell infiltration in the lamina propria (Figure 3C and 3D). In treatment group 2 (gestational diabetic group), 50% of the samples presented dense hyphae (score +2) that could be categorized as severe oral candidiasis. Gestational diabetics often suffer severe oral candidiasis perhaps caused by a compromised immune system resulting from infiltration by T lymphocyte cells of the pancreatic gland that will destroy pancreatic beta cells.¹⁸ Another factor determining the severity of oral candidiasis in this group was the presence of glucose in the saliva which was deposited in the mucosa thus providing the food

necessary for candidal growth. On the other hand, salivary flow rate also plays a role in this condition because saliva flow decreases significantly in individuals suffering from diabetes mellitus, a condition known as xerostomia.

Frequent thirst (polydipsia), dry mouth and binge eating (polyphagia) are characteristics of individuals with diabetes mellitus who demonstrate poor glycemic control which can result in increased diuresis and fluid loss (polyuria). Uncontrolled diabetes causes abnormal defense cell function. Polymorphonuclear leukocytes (PMN) constitute the main defense cells; neutrophils, monocytes and macrophages, in the periodontium. Diabetics typically exhibit the main defense against cell defects because of the imbalance between chemotaxis and phagocytosis which causes sufferers of diabetes mellitus to be more susceptible to infection by *C. albicans*.¹⁹

Through the research reported here, it indicated that the oral candidiasis afflicting the members of treatment group 2 (gestational diabetes) was more severe than that affecting those in group 1 (diabetes type 1/DM 1). This can be attributed to elevated levels of estrogen during pregnancy which will, in turn, produced high levels of glycogen. The increase in glycogen provides a sufficient carbon source for the growth of *C. albicans*.⁷ On the other hand, in treatment group 2 (gestational diabetes) 25% of the sample suffered from mild oral candidiasis, while a further 25% were found to be uninfected. This contrast in infection was possibly due to differences in the respective resistance of members of the sample.

The samples who suffered from oral candidiasis had >400 CFU/ml *C. albicans* whereas, in general, the normal number of *C. albicans* is <400 CFU/ml.^{20,21} Table 3 contains the spore counts in SDA culture media which indicated that all members of the sample groups were at risk of developing oral candidiasis. The results showed the number of colonies of *C. albicans* in all sample groups to be > 400 CFU/ml.

The number of spore colonies indicated the risk of developing oral candidiasis, but could not quantify the severity of the condition. The potential risk could arise because the form of yeast-like *C. albicans* colony has a cell wall containing mannoprotein, chitin and glucan. Mannoprotein possesses immunosuppressive properties which enhance the defense of *C. albicans* against the host's immune system. *C. albicans* cells will decompose polysaccharides, proteins and glycoproteins which not only stimulate that system, but also facilitate attachment to the host cells.²¹⁻²³ In addition to adhering to the surface of the epithelium, *C. albicans* penetrates deeply, especially in the cell junction, by forming an infective hyphae.²¹ Under pathogenic conditions, the form of pseudohyphae and hyphae plays an important role in the penetration process compared to the form of the spores. Indeed, the forms of pseudohyphae and hyphae demonstrates a higher penetrative ability than those of spore forms.^{4,21} Candidal hyphae is known to be highly virulent due to the large shape of its hypae which renders it difficult to be engulfed by

macrophage cells (phagocytosis process). Therefore, the body's immune system requires other mechanisms in order to be able to eliminate candidal hyphae in infected tissue. Disorders that result from phagocytosis reduce the capability of PMN. Phagocytosis due to PMN and macrophages can be inhibited by peptides and acid production from the extracellular glycoprotein *C. albicans*.²³

Extracellular *C. albicans* proteins important to virulence include aspartyl proteinases and phospholipases. Penetration by hyphae supported by aspartyl proteinase and phospholipase will cause reduced production of saliva. Aspartyl proteinase suppresses the production of host proteins such as albumin, hemoglobin, keratin, and Immunoglobulin A secretion (IgA) which play a role in the immune system. The pathogenic effects of *C. albicans* will increase acid production followed by reduced sIgA (secretory IgA) ultimately compromising the body's immune response. Aspartyl proteinase is keratolytic, thereby facilitating the penetration of the epidermis by *Candida*. The phospholipase enzyme is one of the virulent factors that contributes to maintaining infection and also to hydrolyzing phospholipid epithelial cell membranes.^{21,22,24}

The contents of Table 3 show that the number of spore colonies in group 2 (GDM) was higher than that of group 1 (DM 1). Therefore, subjects with GDM were considered to be at greater risk of developing oral candidiasis than those with DM 1, a conclusion strengthened by the results contained in Table 2 which indicate that the hyphae scoring in GDM subjects was greater than that in DM 1 subjects. It can be affirmed that subjects with GDM presented more severe oral candidiasis than those with DM 1, perhaps due to hormonal changes in GDM that lead to an increase in the severity of oral candidiasis.^{1,7}

The presence of hyphae could prove useful in predicting the severity of oral candidiasis because, under pathogenic conditions, the form of pseudohyphae and hyphae play an important role in tissue penetration. Penetration by hyphae supported by aspartyl proteinase and phospholipase will result in reduced saliva production. The phospholipase enzyme is also one of the virulent factors that contributes to the severity of infection.²³

From the results of this investigation, it can be supposed that future sufferers of diabetes mellitus should always maintain their oral hygiene and blood sugar levels in order to protect against oral candidiasis infection. Further research could be undertaken to detect hyphae in oral candidiasis infection using Periodic acid-schiff (PAS) staining methods on oral tissue lesions. By employing an ELISA technique, it could also identify the role of enzymes produced by *C. albicans* in the severity of oral candidiasis infection as a means of applying the most effective therapy.

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