The Effect Of Diabetes Mellitus On The Thickness Of Gingival Junctional Epithelium (Study in the Experiment of Caspase-3)

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Abstract

Diabetes mellitus (DM) is a metabolic disorder manifested by abnormally high levels of blood glucose, resulting in hyperglycemia that affects the oral cavity, leading to periodontitis. The junctional epithelium (JE) is the epithelial component of the dento-gingival unit that is in contact with the tooth surface. Apoptosis and proliferation of JE are essential to maintenance JE thickness. Apoptosis is programmed cell death that can be triggered by various signals and is characterized by well-defined morphologic changes and biochemical features. Caspase-3 is involved in the underlying mechanisms of apoptosis, and the activation of caspase-3 is considered to be the final step in many apoptosis pathways. Purpose of this study was to investigate the effect of DM on the expression of caspase-3 and the thickness of JE. Sixteen male Sprague-Dawley rats were used and divided equally into two groups: the diabetic group that injected intraperitoneal by streptozotocin (STZ) and negative control group. Measurements of blood glucose levels were analyzed before and at 2, 4 weeks after STZ injection. In addition, JE thickness and expression of caspase-3 were examined after 2 and 4 weeks. JE was stained by hematoxylin-eosin (H&E) staining for thickness measurement and the immunohistochemistry by using the anti-caspase-3 antibody for caspase-3 expression measurement and examined under light microscope. The results of the present study showed that a decrease of JE thickness and increase of caspase-3 expression were obtained while increasing the diabetic duration. Two ways Anova and Least Significant Difference (LSD) tests indicated a significant difference of JE thickness and caspase-3 expression between all groups except in diabetic group after 2 and 4 weeks. Also, caspase-3 expression in diabetic group after 2 and 4 weeks ($P > 0.05$) were not significantly different. It can be concluded that diabetes mellitus (DM) affected on the thickness and caspase-3 expression of JE. Furthermore, the results suggest that high expression of caspase-3 was associated with the diabetes-induced apoptotic cell-death resulting in reduction of JE thickness.

Keywords: Streptozotocin, diabetes mellitus, junctional epithelium, expression of caspase-3

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder manifested by abnormally high levels of blood glucose, resulting from defects in insulin production, action, or both. The prevalence of diabetes worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030 [1,2]. The characteristic feature of diabetes is an abnormal elevation in blood glucose levels. Diabetes mellitus (DM) leads to long-term damage to various organs, leading to retinopathy, nephropathy, angiopathy, atherosclerosis, periodontitis, and impaired wound healing [2,3,4].

Several methods have been used to induce diabetes mellitus in laboratory animals with success. The method which is more uniformly effective, convenient, simple and widely used is the injection of streptozotocin (STZ). This treatment leads to the degeneration of the Langerhans islets of β-cells [5,6], which causes a rapid and selective destruction of β-cells with resulting hyperglycemia when injected in vivo [7].

JE (JE) is the epithelial component of the dento gingival unit. It contacts directly with the tooth surface [8], located between a non- renewable tooth surface and gingival connective tissue [9]. JE may be regarded as the most interesting structure of the gingiva. An apical migration of JE is one of the first changes of periodontitis [8].

On the other hand, apoptosis is a highly regulated form of programmed cell death. It can be triggered by various signals and is characterized by distinct morphologic changes and biochemical features [10,11,12]. Apoptosis can be modulated by mediators of inflammation. Abnormal apoptosis has been implicated in various pathologies, e.g., cancer, AIDS, Alzheimer's disease, periodontal diseases, and diabetes [13].

Caspase-3 is a major mediator of apoptosis. It plays a crucial in the promotion of all forms of apoptotic cell death 14. Caspase-3 is considered to be the third final step of apoptosis pathways (Porter et al. 1998; Cai et al. 2002Lu et al. 2009)15,16,17. Based on the background of the study, the objectives of this study were to investigate the effects of diabetes mellitus (DM) on the caspase-3 expression and the JE thickness.

2. Material and Method

This research was an animal experimental laboratory study. All of the animal models were supplied from and cared in LPPT Laboratory, Universitas Gadjah Mada. The animal experiment was conducted following the guidelines approved by Ethics Committee, Faculty of Dentistry, Universitas Gadjah Mada (117/KKEP/FKG-UGM/EC/2011).

Sixteen male Sprague-Dawley rats with an average body weight of 140-150 g (60 days old) have been selected randomly and divided into two groups. Rats in diabetic group were injected intraperitoneally by STZ (Sigma, St. Louis, MO, USA) at the dose of 40 mg/kg of the body weight (BW) (n=8). Rats in the negative control group were left untreated (n= 8). Whole blood was taken from the retro-orbital vein of all rats to determine blood glucose levels before

treatment and immediately before terminations at 2 and 4 weeks. Serum was separated using a centrifuge (ALC Centrifugette 4206, Milano, Italy) at 1500 g for 5 minutes. Measurements of blood serum glucose levels were conducted by using a spectrophotometry method. Serum from all samples was placed in a labeled plastic tube and stored at -20ºC until testing.

Rats were euthanized and sacrificed by decapitation. Junctional Epithelium (JE) specimens from the molar area of the maxilla were processed for paraffin embedding. Paraffin blocks were cut in 4 µm thickness then stained with hematoxylin-eosin (HE) and caspase-3 immunostaining using Starr Trek HRP DAB Staining Protocol (Product Biocare). Slides were evaluated under a light microscope (Leica DM-LB, Leica, and Wetzlar, Germany) with 400X magnification. Besides, JE cells which expressed brown color after caspase-3 immunostaining considered showed positive expression.

3. Results

3.1. Measurement of blood glucose level

Blood glucose level before as well as after 2 and 4 weeks STZ injection increased simultaneously with the diabetic duration (Table 1). Blood glucose level was evaluated highest in the diabetic group after 4 weeks (215.63±35.63mg/dL) and the lowest in the negative control group (86.10±15.88mg/dL) before treatment (baseline). Hence, STZ injection promotes hyperglycemia condition. Diabetes Mellitus (DM) was evaluated histologically from pancreas samples. The result showed degeneration and decreasing of β-cell number in the Langerhans islets in the diabetic group and lead to diabetes type II (Fig. 1).

Group	Measurement	Before Injection	After 2 Weeks
Control	Glucose Levels (mg/dl)	86.10 ± 15.88	93.31 ± 5.85
Diabetic	Glucose Levels (mg/dl)	97.13 ± 11.54	182.79 ± 64.93

Table 1. Mean and standard deviation of blood glucose level (mg/dL)

Microscopic examinations of pancreas in diabetic and negative control groups after 2 and 4 weeks (Fig 1) showed that degeneration and depression of β-cell number in the Langerhans islets of diabetic group. These degenerations and reduction of the number in the β-cells were associated with increased apoptosis and led to type II diabetes.

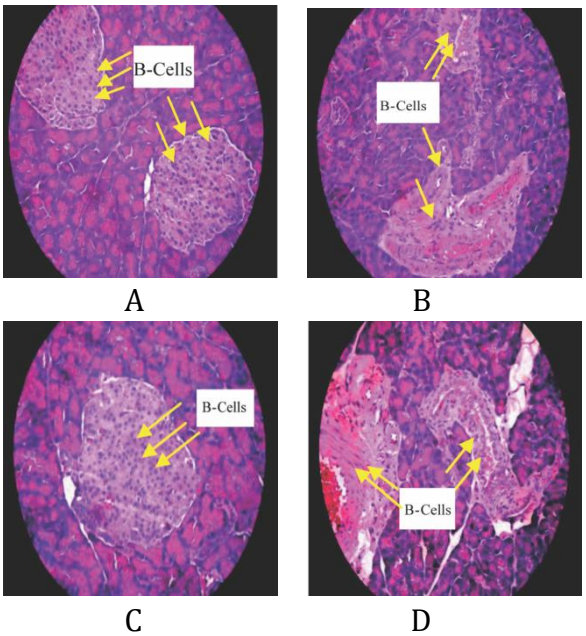
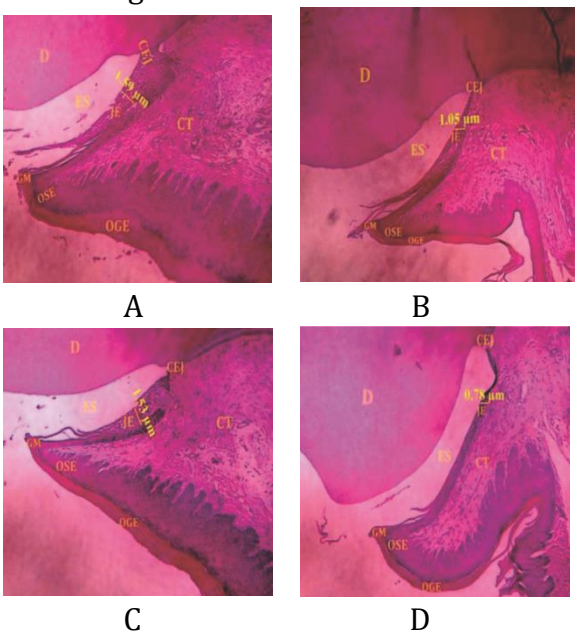


Fig.1. Histological observation of pancreas using hematoxylin-eosin (HE) staining and 400X magnification: A. Normal pancreatic β-cells in the negative control group after 2 weeks B. Degeneration and decreasing of pancreatic β-cells number in the diabetic group after 2 weeks administration of STZ. C. No change of normal pancreatic β-cells in the negative control group after 4 weeks. D. Pancreas swelled. Degenerating and slightly decreasing in the number of pancreatic β-cells in the diabetic group after 4 weeks STZ injection. Arrowheads indicate β cells.

3.2. Measurement of the thickness of JE

We examined JE thickness in control as well as diabetic groups after 2 and 4 weeks. As shown in Figure 2, JE thickness decreased gradually according to increasing of the diabetic duration.



A B C D

Fig.2. JE thickness of the negative control group after 2 weeks (A) and 4 weeks (C). JE thickness reduced in diabetic group at 2 (B) and 4 weeks after injection of STZ (D). JE, Junctional Epithelium; CT, Connective Tissue; D, Dentin; ES, Enamel Space; GM, Gingival Margin; OGE, Oral Gingival Epithelium; OSE, Oral Sulcular Epithelium; CEJ, Cemento-Enamel Junction. Histological figures were taken under a light microscope using 100X magnifications.

We counted from the basal layer facing the connective tissue to suprabasal layer that extending to the tooth surface to evaluate number of JE layers (Table 2). The thickest JE layer was in the negative control group after 2 weeks (1.52 ± 0.12 layers). JE thickness was reduced by the progress of diabetic duration. The less thick JE was observed in the diabetic group after 4 weeks (0.42 ± 0.10 layers). ANOVA test showed significantly different JE thickness between duration and groups. The result of Post-Hoc LSD analysis showed significant difference JE thickness among all groups, except in the negative control groups after 2 and 4 weeks ($p > 0.05$).

Variabel	Junctional Epithelium Thickness (µm)	
	2 weeks	4 Weeks
Control Group	1.52 ± 0.12	1.49 ± 0.22
Diabetic Group	0.74 ± 0.18	0.42 ± 0.10

Table.2. Mean and standard deviation of JE thickness in negative control and diabetic groups after 2 weeks and 4 weeks STZ injection

3.3. Measurement of Caspase-3 expression of JE

We counted number of cells expressing Caspase-3 to determine its possible role in the reduction of JE thickness.

Caspase-3 expression of JE in the negative control groups, diabetic groups after 2 and 4 weeks were increased following diabetic duration (Fig 3).

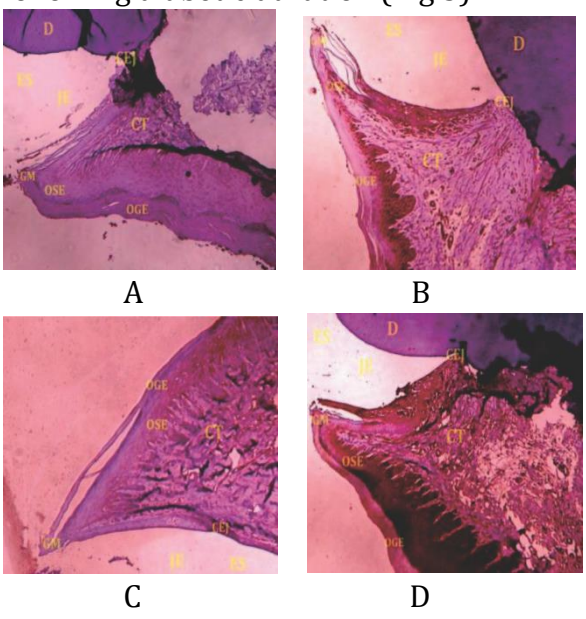


Fig.3. Light microscopic examination of Caspase-3 expression at JE using 100X magnification. Brown color with arrow marker indicates expression of Caspase-3: A.Control group 2 weeks, B. Two weeks after STZ injection groups. C. Negative control group 4 weeks. D. Four weeks after STZ injection groups. JE, Junctional Epithelium; CT, Connective Tissue; D, Dentin; ES, Enamel Space; GM, Gingival Margin; OGE, Oral Gingival Epithelium; OSE, Oral Sulcular Epithelium; CEJ, Cemento-Enamel Junction).

As shown in Fig 3, there was no expression of Caspase-3 in the negative control group after 2 and 4 weeks. However, in the diabetic group, STZ injection promotes diabetic conditions that induced activation of Caspase-3 expression in JE. High expression of Caspase-3 was found in basal cells and suprabasal cells of JE in the diabetic group after 2 weeks and increase slightly in after 4 weeks.

Variabel	Expression of Caspase-3 in JE	
	2 weeks	4 Weeks
Control Group	11.50 ± 16.50	27.00 ± 24.54
Diabetic Group	145.00 ± 47.70	170.75 ± 50.26

Table.3. Mean and standard deviation (Means ± SD) of Caspase-3 expression of JE in negative control and diabetic groups

Cell expressed Caspase-3 was counted in all area of JE (Table 3). It was found that the number of JE cells expressed Caspase-3 was increased along with diabetic duration (Table3). The highest number of cell expressed Caspase-3 was found in diabetic group after 4 weeks (170.75 ± 50.26). The negative control group showed the lowest number of cell expressed Caspase-3 after 2 weeks (11.50 ± 16.50). Post-Hoc LSD test showed significant difference of number of cells expressed Caspase-3 between groups, except in negative control group after 2 and 4 weeks, and in diabetic groups after 2 and 4 weeks ($P > 0.05$).

4. Discussion

The previous study reported intravenously and intraperitoneally administration of STZ induced diabetes in animal models especially rats and mice. Intraperitoneal injection dose ranging from 25 to 100mg/kg STZ successfully induced a

dose-dependent hyperglycemia in rat [5,6]. In this study, we administered a single dose of 40 mg/kg of STZ intraperitoneally to induce diabetic condition based on our preliminary study.

The result of this study showed blood glucose levels increased 2 weeks after STZ injection compared to negative control groups. The slight increase in blood glucose levels indicated chronic hyperglycemia in the diabetic group. Hence, hyperglycemia was considered to be the first stage of induction of diabetes. In agreement with other studies, the result suggests that alterations in blood glucose revealed a dose-response relationship between STZ and the severity of diabetes. Hyperglycemia was observed in rats within 3 days of STZ treatment and within a week to 10 days [7,18,19,20].

Most of studies on experimental diabetes mentioned STZ injection leads an autoimmune process that promotes pancreas swelling then degeneration in Langerhans islet β-cells thus induces diabetes mellitus in the 2-4 days. In this study, the light microscopy evaluation showed degeneration on β-cells Langerhans islet of diabetic rat 2 and 4 weeks after STZ administration. This degeneration, as well as the decrease number β-cells, indicated type II diabetes 5,18,21. This reduction in β-cell number was associated with increased apoptosis mediators [22].

It was found that diabetic condition reduces JE thickness. Since the size of cell populations was determined by the rates of cell proliferation, differentiation, and death by apoptosis[23], we evaluated apoptosis mediator Caspase-3 role. Previous studies showed Caspase-3 plays a crucial and extensive role in the promotion of apoptotic cell death. The activation of Caspase-3 is considered to be the final step in different apoptosis routes. [14,15]. In agreement with the literature, there was an increase of Caspase-3 expression in diabetic groups after 2 and 4 weeks compared to the negative control groups. The data obtained from this study showed that the diabetic group after 4 weeks exhibited the highest expression of Caspase-3 in JE.

Recent studies provide evidence of type I diabetes was associated with elevated levels of systemic markers of inflammation [24]. Diabetes promotes the formation of Advanced Glycation Endproducts (AGEs) and reactive oxygen species (ROS), as well as higher levels of Tumor Necrosis Factor-α (TNF-α), interleukin-6 and oxidative stress. ROS are potent inducers of apoptosis. TNF induces apoptosis by binding to the TNF receptor-1, which has a death domain that triggers the initial events in apoptosis, and by stimulating expression of pro-apoptotic genes [10,25,26]. In this study, all of these factors might be affected on JE and lead to tissue destruction via direct effects on basal and suprabasal cells, or indirectly through promoting inflammation and apoptosis of these matrix-producing cells. Thus, by enhancing the production of ROS, TNF, and AGEs, diabetes may impair the tissue healing response and decrease the thickness of JE.

In recent studies, diabetes causes thickening of vascular basement

membranes thus reducing tissue nutrition and migration of leukocytes. Tissue maturation and homeostasis appear to be affected by glucose levels [20,27,28]. The histological findings of the present study indicated that the reducing tissue nutrition and leukocytes migration might affect JE. This phenomenon indicated by a slight decrease of JE thickness in basal layer facing the connective tissue and the suprabasal layer extending to the tooth surface.

It was found that diabetes was also able to induce apoptotic cell-death in JE by activating Caspase-3. Several aspects of diabetes could enhance apoptosis. Long-term complications of diabetes are mainly associated with prolonged hyperglycemia. Several studies have explained the biochemical pathways that lead to vascular complications. These include activation of the polyol pathway, leading to the formation of AGEs and oxidative stress along with an accumulation of diacylglycerol and activation of protein kinase C [10,24]. There are several mechanisms by which AGEs can enhance apoptosis: the direct activation of caspase activity and indirect pathways that increase oxidative stress or the expression of pro-apoptotic genes that regulate apoptosis 29. AGEs products can affect connective tissue by promoting apoptosis of matrix-producing cells. The number of cells capable of producing matrix would be reduced because of apoptosis [10]. Extracellular matrix production has been observed even in diabetic conditions [29]. Most importantly, apoptosis and high expression of Caspase-3 induced by high levels of glucose were suppressed by a Caspase-3–specific inhibitor [12,16,25].

5. Conclusion

Diabetes mellitus influences the reduction of junctional epithelium thickness in rat models and increases the Caspase-3 expression of JE.

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7. References

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