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Research Article
Immunohistochemical Expression of Insulin and Glucagon, Superoxide Dismutase and Catalase Activity in Pancreas in Hyperglycaemia Condition

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Abstract

Background and Objective: Hyperglycemia leads to changes on a cellular level that potentially accelerates to induce cell damage. This study investigated the effect of chronic hyperglycemia to superoxida dismutase and catalase activity and expression of insulin and glucagon by immunohistochemistry in rat pancreas. **Methodology:** Sprague dawley albino male rats (200-225 g) were divided into 2 groups. Group I was normal control, group II was hyperglycemia control. Observations were carried on the levels of blood glucose, superoxida dismutase (SOD) and catalase activities, malondialdehyde (MDA) levels, β and α-cells pancreatic immunohistochemically and histology analysis. **Results:** The results showed that rats experienced hyperglycemia from the 4th week and suffered hyperglycemia for 1 month with final glucose level was 139.5±5.2 mg dL^{-1}, whereas, the glucose level of the control group was 97.8±4.3 mg dL^{-1}. Hyperglycemia caused pathological changes in pancreatic tissue, namely increased malondialdehyde level at 31.85±5.69 pmol g^{-1}, while MDA levels in control group only at 22.94±3.82 pmol g^{-1}. The SOD antioxidant enzymes and catalase activities on control groups were at 31.37±3.60 and 0.85±0.08 U g^{-1}, respectively, then they decreased to 21.18±2.34 and 0.67±0.03 U g^{-1} in the hyperglycemia group. Total percentages of β and α-cells in control group were 87.30±6.70 and 48.66±2.64, respectively, then they decreased to 74.54±4.35 and 41.96±2.56 in the hyperglycemia group. In hyperglycemia group, degeneration with mild degree was observed in the pancreatic cells islet of langerhans. **Conclusion:** Hyperglycemia lead to pathological conditions in rat's pancreatic tissue with increased levels of malonaldehyde (MDA), decreased SOD and catalase activity, reduced expression of insulin in β-cells and nucleus of β-cell showed picnotics.

Key words: Hyperglycemia, β and α-cell, malondialdehyde, superoxida dismutase, catalase

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oxidative stress seems cannot to be avoided for humans or animals, even through it depends on the causes and the severity of the stress. In conditions of enhanced metabolism or increased mitochondrial activity lots of tissue are continually as target from free radicals[1,2]. The fact that glucotoxicity might be associated to oxidative stress and that are proposed to play a role in diabetes and atherosclerosis[2-4]. When the excessive free radicals would be very dangerous because it can cause cells damage. There are several factors that cause oxidative stress conditions including food consumption, lifestyle, metabolic abnormalities, antioxidant enzyme deficiency, radiation, drugs, inflammation, pollutants and others[5].

Most of the population in the world, including in Indonesia, highly consume carbohydrates. Excess of glucose in the blood, or often called as hyperglycemia is a starting point for free radicals formation in the body by various biochemical reactions, such as, the electron transport chain in mitochondria, glucose auto oxidation and glycosylated proteins[3,6].

Diabetes Mellitus (DM) is a metabolic disorder affecting about 5-10% of the world's population[7]. Diabetes mellitus is a metabolic disorder that is one of them caused by excessive consumption of carbohydrates and that is characterized by hyperglycemia[8] and the presence of hyperglycemia is associated with increased morbidity and mortality in individuals[9].

Excessive carbohydrate consumption in a long period can lead to hyperglycemia condition. High glucose levels associated with the onset of oxidative stress. According to Yang *et al.*[10] an elevated level of glucose has been found in the blood of hyperglycemia and diabetes and found that hyperglycemia induced extracellular ROS increase and resulted in neuronal apoptosis.

Hyperglycemia induces formation of anion superoxide by the electron transport chain in mitochondria[3,6], generates ROS by advanced glycation end products (AGEs) formation and alter the metabolic activity of the polyol pathway[11]. In addition, according to Aronson[12], there is a molecular mechanism of cell damage caused by hyperglycemia, including it increases inflammatory by inducing cytokine secretion, AGE products (advanced glycation end products) formation, activation of protein kinase C (PKC) and hexosamine pathway flux.

At high concentrations, free radical can be important mediators of damage to cell structures, nucleic acids, lipids and proteins[13]. The experimental evidence show that Reactive Oxygen Species (ROS) contribute to cell and tissue dysfunction and damage caused by glucolipotoxicity in diabetes[1]. In contrast to most other mammalian cell types, in β-cells increased glucose concentration stimulates a rapid and proportional increase in glycolytic flux and TCA cycle, which can lead to an enhancement of ROS production[1,14].

Glucose itself is capable of generating reactive oxygen species (ROS) in β-cells so it can be stated that oxidative stress-induced glucose or also known as glucose toxicity[15]. Potential glucose-related pathways through which ROS can be formed include autoxidation, oxidative phosphorylation, glycosylation and the glucosamine pathways[15,16].

High sugar consumption is also associated with an increase in free radical generation leading to damage of fats, protein and DNA[17]. In addition, Campos[18] also said that chronic hyperglycemia causes pathological changes in pancreatic tissue and complications of other tissues that can effect diseases including: Diabetic, heart diseases, atherosclerosis, neuropathy and retinopathy[13,18]. The purpose of this study is therefore designed to investigate effect of chronic hyperglycemia on superoxide dismutase and catalase activity and expression of insulin and glucagon by immunohistochemistry in rat pancreas. Basically, we would like to inform that the danger of hyperglycemia condition which can impact on the pathology of pancreatic tissue.

MATERIALS AND METHODS

Preparation of the experimental animals: In this study sprague dawley albino male rats (200-225 g) were obtained from the experimental animal facility of The Faculty of Veterinary Medicine, Udayana University. Rats were divided randomly and it were housed in 25×15×45 cm cage. All rats were adaptation laboratory conditions for 10 days at a temperature of 25-30°C and a relative humidity of 55-65%. During the adaptation and treatment, rats were given standard pellet diet. The experimental protocols applied to rats were based on guidelines for the care and use of animals in accordance to the chapter 7.8 on Use of Animals in Research and Education of Terrestrial Animal Health Code of the World Organization for Animals Health and ethical clearance of the local ethical committee for the use of animals in research and education at Udayana University (Ref No.135a/KE-PH/IV).

Experimental design: Rats were randomly divided into two groups. The control group (K0) (n = 9) and the treated hyperglycemia group (HG) (n = 9). Rats in hyperglycemia group were given 80% sucrose solution (w/v) (based on a preliminary study) 2 mL orally twice a day for 56 days

(8 weeks). Fasting blood glucose levels of the rats were analyzed every week for 56 days (8 weeks). At the end of the study, the rats were necropsied by euthanasia with 0.1 mL/body doses of 100 mg mL^{-1} ketamine-HCl. Immediately after the necropsy, pancreatic tissue was taken for analysis malondialdehide levels superoxide dismutase and catalase activities, α and β-cells in immunohistochemically staining and histopathology of the pancreatic us in HE staining.

Measurement of blood glucose levels: Measurement of blood glucose level was done on days 0 (initial data base) and every 1 week for 56 days (8 weeks). Blood glucose was determined by using blood glucose testing system (glucose strip method), a blood glucose test meter. Blood was taken from the tail vein of the rats and was placed on the strip on the glucometer test.

Analysis of malondialdehyde (MDA): The MDA level was determined by adapting method of Singh *et al.*[19]. A total of 0.5 mL of the sample (lysate pancreas) [or standard] added with 2 mL of 0.25 N HCl cold mixed solution containing 15% thricloroacetic (TCA), 0.38% thiobarbituric acid (TBA) and 0.5% butylated hydroxytoluene (BHT). The mixture solution was heated to 80°C for 1 h and before then it was. The TEP (1,1,3,3-tetraethoxypropane) was used as the standard solution. The value of absorbance solution was measured at l532 nm.

Analysis of superoxide dismutase (SOD): The SOD activity was assayed adapting to the method of Wijeratne *et al.*[20], with some modifications. A total of 60 µL pancreatic lysate was mixed with 2.7 mL sodium carbonate buffer containing 0.1 mL EDTA (ethylene diamine tetraacetic acid), 0.06 mL 10 mM xanthine, 0.03 mL 0.5% Bovine Serum Albumin (BSA) and 0.03 mL 2.5 mM nitroblue tetrazolium (NBT). Then, this mixed solution added 0.04 units xanthine oxidase. The absorbance was measured at λ 560 nm. The SOD activity (%) has calculated with the following equation:

$$\left[1-\frac{A}{B}\right]\times 100$$

where, A is absorbance of the sample and B is absorbance of the control.

Analysis of catalase: Catalase activity was analyzed adapting to the method of Rice-Evans *et al.*[21]. The analysis was done by mixing 1 mL of homogenate samples in 5 mL of 0.05 M phosphate buffer pH 7 in the test tube and homogenized, then added 2 mL of 0.2 M H_2O_2 and incubated for 30 min at room temperature. The mixture was taken 1 mL and added to 2 mL of 5% $K_2Cr_2O_7$ solution and heated in boiling water for 10 min and the absorbance was measured at λ 570 nm. About 1 M H_2O_2 was used for this standard solutions.

Analysis of insulin and glucagon expression in β and α-cells: Determination of α and β-cells of the iselet of langerhans pancreatic was done using immunohistochemical staining with the two-stage indirect method procedure that refered to the manufacturer's instructions in the kit. Anti-insulin (Monoclonal Anti-insulin, No. I2018, Sigma) and anti-glucagon (Monoclonal Anti-glucagon, No. G2654, Sigma) antibodies were used for primary antibodies. The DAKO LSAB kit+System-HRP (Lot 10.07892 million; Ref. K0679) were also used as the kit. Briefly, the method was done as follows, preparations tissue was soaked with 3% H_2O_2 for 15 min, washed with PBS and then soaked with 1.5% skim milk for 20 min. The preparations were washed with PBS and given primary anti-insulin or anti-glucagon antibodies and then incubated for 3 h at room temperature. Next, the preparation was washed and given a secondary antibody and incubated at room temperature for 30 min. Then washed with PBS and soaked with streptavidin-HRP and incubated for 20 min at room temperature. Preparations were washed with PBS and soaked with diaminobenzidine (DAB) until brown color appeared. Preparations were washed, then countarstained with meyer-hematoxyline. Thereafter, preparations observed under a microscope. Expression of β-cell insulin and α-cell glucagon in pancreatic islets of langerhans using IHC H-score modified[22].

Histopathological analysis: Pancreatic tissues were fixed in 10% buffered formalin solution for at least 24 h. Samples were cut into smaller pieces and placed in tissue cassettes for further processed, dehydration, clearing, infiltration, embedding, cutting and HE staining. Preparations that had already stained than were observed under a light microscope with a magnification of 400 times. The observation was made to evaluate the general histologically structure of tissue including the changes that occured.

Statitical analysis: Superoxide dismutase, catalase, malondialdehyde, expression of insulin and glucagon data of the pancreas control group and the hyperglycemia group were compared and analyzed by using unpaired T-test. All of

the data was tested for homogeneity and normal distribution using Levene's test and one-sample Kolmogorov-Smirnov test, respectively. Characteristics of the study group were expressed as Mean+Standard Deviation for normal distribution. The analysis was carried out in SPSS software, (SPSS, Chicago, IL, USA) version 12.0, at a significance level set at $p<0.05$. While the data for pancreatic histopathology were analyzed descriptively.

RESULTS AND DISCUSSION

Results of blood glucose levels: The results analysis of blood glucose levels in rats for 56 days (8 weeks) are shown in Table 1 and Fig. 1. The data in Table 1, blood glucose levels in the control group shown relatively similar, which ranged from 90.7-97.8 mg dL^{-1} and blood glucose control groups were not significantly different ($p>0.05$). Blood glucose levels on rats hyperglycemia group, showed the initial glucose levels (week 0) at 94.7 mg dL^{-1} and after 8 weeks the blood glucose levels reached 139.9 mg dL^{-1}.

Blood glucose levels on hyperglycemia groups begin to increase at 4 weeks after treatment. Furthermore, the blood glucose levels of rats were continually increased until at the end of the experiment period (Fig. 1).

Results of malondialdehyde (MDA) levels: Malondialdehyde is the end product of lipid peroxidation. High level of MDA indirectly shows a high number of free radicals in tissues. The analysis results of malondialdehyde level in rats are shown in Table 2.

Malondialdehyde (MDA) levels on rat pancreatic control and hyperglycemia group an average of 22.94 pmol g^{-1} and of 31.85 pmol g^{-1}, respectively. The MDA levels of hyperglycemia treatment was significantly higher ($p>0.05$) when compared to control. This suggests that the conditions of hyperglycemia has caused damage to the pancreatic tissue which was characterized with the MDA level increased. Pancreatic tissue was damage due to the conditions of hyperglycemia, in which β-cells continuously producing insulin and then causing β-cells exhaustion. Beta-cell exhaustion would be followed by damage to the cellular components, including lipid peroxidation. This was characterized by increasing of the MDA levels.

Results of superoxide dismutase and catalase activity: The analysis results of superoxida dismutase (SOD) and catalase (CAT) activity in pancreatic tissue are shown in Table 2. Intracellular antioxidant enzymes such as SOD, catalase and glutathione peroxidase are endogenous antioxidants that act to protect body cells against oxidative damage. Table 2 shows that, SOD activities in control group was higher than catalase enzyme pancreatic. In the hyperglycemic group, the activity of SOD and catalase were both significantly lower than that of the control group ($p<0.05$), 32.5 and 21.6%, respectively. These results show that hyperglycemia may be associated with increased metabolic reactions that produce reactive oxygen species.

Results of insulin and glucagon expression in β and α-cells: The results of the expression of β-cells insulin and α-cell glucagon analysis are presented in Table 3 and Fig. 2 and 3. Table 3 shows that, the expression of insulin in β-cells islets of langerhans in hyperglycemia group lower compared to the expression in the control group, although they were not significantly different ($p>0.05$). Similarly, the expression of α-cells in hyperglycemia group was not significantly different ($p>0.05$) compared to the control group.

The results of immunohistochemical staining for insulin in β-cell and glucagon in α-cells on islets of langerhans are shown in Fig. 2 and 3. Insulin and glucagon are secreted by β and α-cells in islet of langerhans, respectively and their are expresded in the cytoplasm and nucleus. Insulin and glucagon

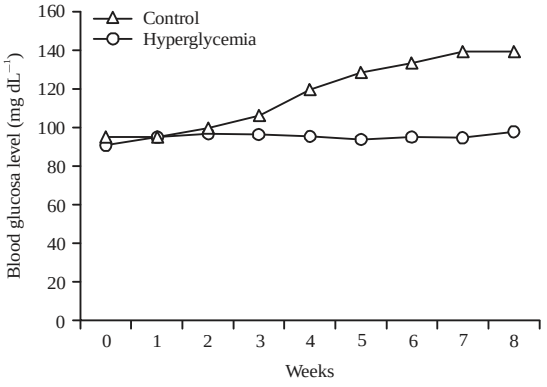

Fig. 1: Profile of blood glucose levels on rats for 8 weeks of treatment

Table 1: Average blood glucose levels on rats for 8 weeks of treatment

	Blood glucose levels (mg dL^{-1}) for 8 weeks								
Treatment	0	1	2	3	4	5	6	7	8
Control	90.7±2.9	94.7±5.4	96.7±3.9	96.2±3.3	95.3±3.2	93.9±3.5	94.9±4.5	94.7±3.7	97.8±4.3
Hyperglicemia	94.7±5.2	95.0±4.6	99.5±6.3	106.2±4.4	119.5±3.9	128.5±4.9	133.4±4.7	138.8±3.3	139.5±5.2

Values are expressed as Mean±SD

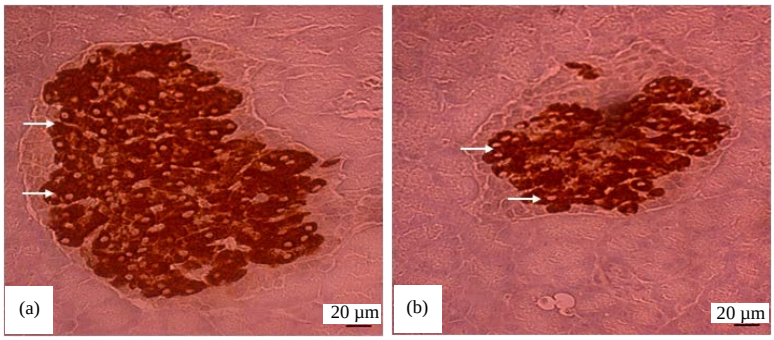

Fig. 2(a-b): Results of immunohistochemistry test (400x) showed expressions of insulin in β-cells (arrow: Brown in the cytoplasm and nucleus of cells) on the (a) Islets of langerhans pancreas control and (b) Hyperglycemia on the 56th day

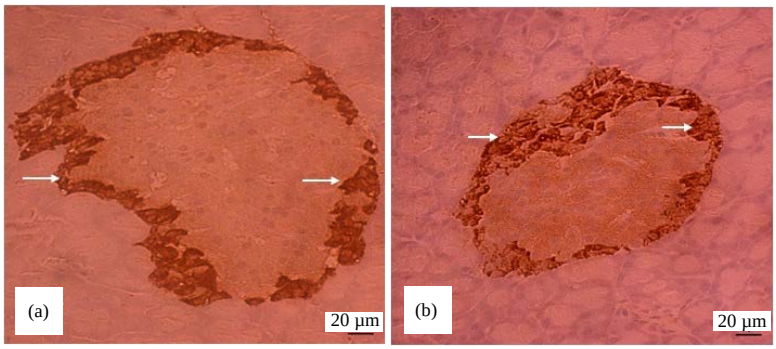

Fig. 3(a-b): Results of immunohistochemistry test (400x) showed expressions of glucagon in α-cells (arrow: Brown in the cytoplasm and nucleus of cells) on the (a) Islets of langerhans pancreas control and (b) Hyperglycemia on the 56th day

Table 2: Malondialdehyde (MDA) levels, superoxide dismutase (SOD) and catalase (CAT) activity in rats pancreas on the 56th day

Treatment	MDA levels (pmol g^{-1})	SOD activity (IU mL^{-1})	CAT activity (IU mL^{-1})
Control	22.94±3.82[a*]	31.37±3.60[a]	0.85±0.08[a]
Hyperglycemia	31.85±5.69[b]	21.18±2.34[b]	0.67±0.03[b]

*Value which followed by different letters in the same row indicate significantly different ($p<0.05$)

Table 3: Percentage of insulin and glucagon expression in α and β-cells of rats pancreas on the 56th day

Treatment	Expresion of insulin (β-cells %)	Expresion of glucagon (α-cells %)
Control	87.30±6.70[a]	48.66±2.64[a*]
Hyperglycemia	74.54±4.35[a]	41.96±2.06[a]

*Value which followed by different letters in the same row indicate significantly different ($p<0.05$)

were indicated to positive reaction on immunohistochemical staining by showing brown color. In general, β and α-cells in control and hyperglycemia groups did not change significantly. Beta-cells are located in a central core and surrounded by α-cells on islet of langerhans.

Histological analysis: In general, the results of microscopic observation of pancreatic tissue picture shows that Islets of langerhans appears highly obvious with more intense color than the surrounding exocrine glands (Fig. 4). The results of microscopic observation in the control group showed images islets of langerhans, both in terms of shape, size and mass cells of considerable quantity.

In normal rat, blood glucose levels are varies. According to Ramesh and Pugalendi[23], the blood glucose levels on rat ranged from 74.35-84.85 mg dL^{-1}, while Gutierrez *et al.*[24] reported normal blood glucose levels ranged form 91.19-95.41 mg dL^{-1}. This study showed normal levels of blood glucose on control rat group ranged from 90.7-97.8 mg dL^{-1}. While, the blood glucose levels on hyperglycemia rat group, began to increase from 4-8 weeks of the period and the highest glucose concentration reached at 139.5 mg dL^{-1}. This means that the blood glucose levels on rat are above the limit of normal values which indicates that the rats were hyperglycemia during the experiment.

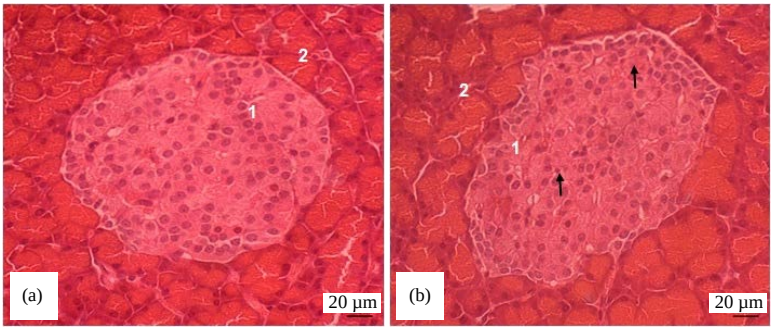

Fig. 4(a-b): Results of histology HE staining on (a) Islets of langerhans pancreatic control and (b) Hyperglycaemia visible cell nucleus undergo picnotics (arrow) on the 56th day. 1: Islets of langerhans and 2: Exocrine of glands

Rats were hyperglycemia after 4 weeks treatment and during the next 4 weeks until the end of the study blood glucose levels were gradually increased (Fig. 1). This means administration of 80% sucrose solution two times a day have been able to increase blood glucose levels on rats for 4 weeks observation.

Robertson[6] stated that, hyperglycemia is the starting point of free radicals formation through various biochemical reactions. Hyperglycemia can activate four glucose metabolic pathways which can increase free radical formation in mitochondria[25]. Hyperglycemia related ROS can generate biochemical reactions including autoxidation, oxidative phosphorylation, glycosylation and glucosamine pathways[11,16,25].

According to Wolff and Dean[16], glucose can undergo autooxidation reaction produces hydroxyl radicals ($OH^{\bullet}$). Reaction process through enolysation glycerides-3P to form enedyol. Furthermore enediol, reacts with Fe^{3+} produce enedyol radical. Meanwhile, Fe^{2+} ions result of reduction of Fe^{3+} will react with hydrogen peroxide to produce hydroxyl radicals. Free radicals that formed (enedyol radicals, hydroxyl radicals and superoxide anion) potentially cause a reaction between carbohydrates with protein glycosylation which contribute to destruct of cell membranes. Some research indicate that on diabetic condition intracellular antioxidant enzyme levels were decreased but MDA levels were increased.

Robertson *et al.*[15] have shown that chronic hyperglycemia can increase reactive oxygen species levels in β-cell. The use of animal models with deteriorating glucose control in type 2 diabetes caused oxidative stress in β-cells. Reactive oxygen species was correlated positively with the increased markers of cell damage. Ihara *et al.*[26] analysed markers of tissue damage that was 8-hydroxy-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins in high levels of type 2 diabetic rat.

Malondialdehyde is a compound produced from unsaturated fatty acid oxidation and its used as a biomarker of cell membrane damage caused by free radicals[27]. Studies on rats and mice as animal models of diabetic showed that there was an increase level of malondialdehyde in the blood[28].

Free radicals are produced during hyperglycemia will neutralized by antioxidant enzymes to form a more stable compound. The primary antioxidant enzymes are superoxida dismutase, gluthathione peroxidase and catalase[29,30]. The SOD dismutates catalyse superoxide anion ($O_2^{\bullet}$) radicals reactions to form H_2O_2 and O_2, while catalase catalyses the breakdown of hydrogen peroxide to be H_2O and O_2[13,30]. Table 2 shows that treatment of hyperglycemia increased MDA products to 38.8% and reduced the level of SOD to 32.5% and catalase to 21.6%. This indicating that hyperglycemia might have potential contribution on cell damage.

Free radicals that have been generated during hyperglycemia reflected in high MDA levels pancreatic and these will increase the use SOD and catalase pancreatic so that the both enzymes activity were decreased. In normal pancreatic, the major antioxidant enzyme levels both SOD, catalase and GPx concentrations present in relatively fewer when compared with other tissues such as liver, kidney and muscle[26] so that the pancreatic is very sensitive to free radical attack. Poitout and Robertson[3] and Tiedge *et al.*[31] reported that β-cells have very low levels of antioxidant enzymes compared with other tissues. Suggest that the β-cells is particularly at risk for oxidative stress.

Chronic hyperglycemia condition may result in excessive production of free radicals from the auto-oxidation of glucose, protein progression and change of the oxidants and antioxidants the body and was occur a change in the balance of oxidants and antioxidants balance in the body. Excessive free radical formation in diabetics can lead to a decrease in enzymatic antioxidant capacity of the body and tissue damage.

Both β and α-cell numbers were not different in the two treatment groups. According to Steiner *et al.*[32], distribution of β-cells in the islets of langerhans are in central core, surrounded by numbers α-cell, γ-cells, δ-cell and PP-cells.

Pancreatic islets of langerhans consist of several endocrine cell types. Based on endocrine cell types, approximately β-cells representing 60-80% of the total cells in the islet are in central core then surrounded by a number of α-cells (10-20% of cells), δ-cells (<10 of cells) and PP-cells (<1% of cells)[32-34]. The main hormones that are produced by cells type β-cells, α-cell, δ-cells and PP-cells namely insulin, glucagon, somatostatin and pancreatic polypeptide, respectively. In relation to the levels of antioxidant enzymes in the pancreatic is relatively less compared with other organs[15], then components the islets of langerhans cells are very sensitive on free radical attack. According to Sreenan *et al.*[35], in diabetic mice the components of the islets of langerhans cells will morphological change. It affets the decreased numbers of islets and severely reduced number of β-cells. In the hyperglycemic group showed damage in the cells which were degeneration with mild degree and some cells were picnotic in the nucleus cells (Fig. 4). This occurred due to conditions of hyperglycemia causes pancreatic cells, particularly β-cell experienced because of non stop function to produce and excrete insulin. When the condition occurred cronically and supported additional fatigue factor can cause stress and lead to faster cell having damage. In addition, the condition of hyperglycemia can also cause oxidative stress[25], due to excessive production of free radicals. Free radicals can attack surrounding body cells, including cells in pancreatic that were having damaged.

Basic research on hyperglicemia effects on pancreas gives valuable information on how important is to prevent body from chronic hyperglicemia condition. The chronic condition has been proven to cause decreased antioxidant pancreatic enzymes, decreased insulin expression and pancreatic cells experienced picnotic. Therefore, for diabetic type II patients, its important to maintain glucose level as reported by Inzucchi[36].

Several studies[37-39] supported and demonstrated that hyperglycemia in diabetic animals significantly decrease the endogenous antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and increase level of malondialdehyde. This is in accordance with the results on the effect of hiperglcemia on malondialdehyde levels, superoxide dismutase and catalase activity are presented in Table 2.

Administration using sucrose solution is a new treatment, which was used to cause hiperglycemia in this study. While, other studies were mainly using diabetogenic chemistry in causing hiperglycemia, for example alloxan. Other important finding are the immunohisthochemical expression of insulin and glucagon in α and β-cells in pancreas, which has not yet reported with other studies. Additionally, this study also found the effect of the administration i.e., the damage of the pancreatic tissue cells; which were histopathologically picnotic.

This study is basically very important and interesting for study of research development of treatments in order to decrease blood glucose level and antioxidant compounds in order to minimize the free radical effects caused by hyperglycemia condition.

CONCLUSION

The present study have indicated that hyperglycemia causes pathological conditions in rat's pancreatic tissue with increased levels of malonaldehyde (MDA), decreased of enzymes SOD and catalase activity, reduced expression of insulin in β-cells and nucleus of β-cells showed picnotics.

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