Hayati, Juni 2003, hlm. 61-65
ISSN 0854-8587

Vol. 10, No. 2

Immunohistochemical Study of Superoxide Dismutase in the Liver of Alloxan Diabetes Mellitus Macaques

TUTIK WRESDIYATI[1*], R. P. AGUS LELANA[2], I KETUT MUDITE ADNYANE[1], KHALIDA NOOR[1]

[1]*Laboratory of Histology, Department of Anatomy,* [2]*Department of Clinic, Faculty of Veterinary Medicine, Bogor Agricultural University, IPB Darmaga, Bogor 16001*

Diterima 6 Agustus 2002/Disetujui 8 November 2002

Alloxan exhibit the most potent diabetogenity and are used for induction of experimental diabetes mellitus. Many studies reported an understanding of mechanism of diabetogenic agent causes diabetes. However, only a few report on superoxide dismutase in the tissues of experimental alloxan diabetic condition. One of the intracellular antioxidant - copper, zinc-superoxide dismutase (Cu,Zn-SOD) was immunohistochemically studied in the liver of alloxan diabetes mellitus Macaques (*Macaca fascicularis*). A total of 12 male *Macaca fascicularis* were used for this experiment. They were divided into three groups, control group, non-insulin-dependent diabetes mellitus (NIDDM) group, and insulin-dependent diabetes mellitus (IDDM) group. The hyperglycemic condition was obtained by alloxan induction to alter the β-cells of pancreas. The reaction products of Cu,Zn-SOD were qualitatively observed in the cytoplasm of hepatocytes, as well as quantitatively observation in the nucleus of the cells. Both qualitatively and quantitatively observation showed that the enzyme decreased in the liver of both diabetes groups when compared to the control group. The decrease of the SOD was more clearly shown in the IDDM group than in the NIDDM group. The decrease of the enzyme was also showed by the decrease in percentage of positive-cells, and increase in percentage of negative-cells to the total hepatocytes in the liver tissues of both diabetes mellitus groups as compared to the control group. These results suggested that diabetes mellitus condition may increase oxygen-free radical and therefore it decreased the content of Cu,Zn-SOD in the liver tissues.

INTRODUCTION

Antioxidants as free-radical scavengers play an important role in the protection of cells against oxidative stress and maintain a balance between the various toxic oxygen species (Touati 1992). The protection can be done in several ways such as by prevention, stopping or decreasing of oxidations (Schuler 1990), as well as by catalyzing free radicals by intracellular antioxidant enzymes (Mates *et al.* 1999).

The intracellular antioxidant enzymes comprise catalase, glutathione peroxidase, and three isoforms of superoxide dismutase (SOD); copper, zinc (Cu,Zn)-SOD, manganese (Mn)-SOD, and iron (Fe)-SOD. The SOD provides a primary defense against superoxide anion radical generated intracellularly. The distribution of SOD in rat tissues was reported immunohistochemically by Dobashi *et al.* (1989), and immunocytochemically by Wresdiyati and Makita (1998). The enzyme was localized in the liver, lung, kidney, intestine, and heart of rats.

Increased levels of the active oxygen species, free radical, create a situation known as oxidative stress, which lead to a variety of biochemical and physiological lesions often resulting in metabolic impairment and cell death. This highly reactive oxygen can readily react with various biological macromolecules such as DNA, proteins, lipids, and cause protein destruction. The lesions in turn lead to various diseases and degenerative process such as aging and carcinogenesis in humans and animals (Ames & Shigenaga 1992).

Hyperglycemia is a common feature of all forms of diabetes mellitus and has been implicated in a number of diabetes-related conditions, including retinopathy, nephropathy, neuropathy, and vasculopathy (Cerami *et al.* 1988). Diabetes mellitus occurs in nonhuman primates with approximately the same frequency as in human beings (Howard 1982, Wagner *et al.* 1996). Many metabolic, hormonal, and pathologic abnormalities common to human diabetic subjects have been reported in nonhuman primates, making them excellent models of the disease (Howard & Yasuda 1990, O'Briend *et al.* 1996).

Reactive oxygen species (ROS), which induce oxidative damage, are reported to have been correlated to several diseases, such as cancer, heart injury, aging, and diabetes mellitus (Freisleben 2001). Matkovics *et al.* (1997) reported that in alloxan diabetic rat tissues, the oxidative stress is enhanced. Kakkar *et al.* (1998) also reported an increased oxidative stress in rat liver and pancreas during progression of streptozotocin-induced diabetes. The oxidative stress may be caused by the highly blood glucose level, that cannot be used or stored in the tissues, and increase of the oxidation process to produce energy sources. Oxidative damage was also reported in the non-insulin-dependent diabetes mellitus patients (Aguirre *et al.* 1998, Leinonen *et al.* 1997). So far, there are a few reports on intracellular antioxidant especially Cu,Zn-SOD, immunohistochemically, in the tissues of experimental alloxan diabetic macaques (*Macaca fascicularis*).

*Penulis untuk korespondensi, Tel. +62-251-626064, Fax. +62-251-629464, E-mail: astawan@indo.net.id

This study was designed to reveal the intracellular antioxidant status, immunohistochemically, especially Cu,Zn-SOD in the liver tissues of experimental diabetes mellitus in *Macaca fascicularis*. The observations were done qualitatively in the cytoplasm of hepatocytes, as well as quantitatively in the nucleus of the cells, which were expected to give different degrees of reaction products to Cu,Zn-SOD.

MATERIALS AND METHODS

Treatment of Animals. A total of 12 male Macaques (*Macaca fascicularis*) were used for this study. The animals were obtained from 28 male (6-7 years old, 3-5 kg body weight) experimental diabetes mellitus macaques using alloxan induction. They were divided into three groups; control group (blood glucose <120 mg%), non-insulin-dependent diabetes mellitus group/NIDDM (blood glucose 120-350 mg%), and insulin-dependent diabetes mellitus group/ IDDM (blood glucose >350 mg%). Preparation of the experimental animals was done at the Primate Research Center, Bogor Agricultural University.

Tissue Preparation. After the treatment, all animals were necropsied following perfusion via their heart. Pieces of tissues from liver were fixed with 4% paraformaldehyde for 3 days. Tissues were dehydrated through a graded ethanol series, and embedded in paraffin. Specimens were cut into 4 μm-thick sections and subjected to immunohistochemical study of Cu,Zn-SOD.

Immunohistochemistry. SOD was localized immunohistochemically as described previously (Dobashi *et al.* 1989) with a modification. The tissue sections were washed for 15 min with 3 changes of phosphate buffer saline (PBS) between each step. After deparaffinization and rehydration, the tissue sections were exposed to 3% H_2O_2 for 10 min to inactivate endogenous peroxidase activity and then to 10% normal goat serum to block nonspecific binding. Following rinsing with PBS, the tissue sections were incubated in primary antibody of copper,zinc-superoxide dismutase (Cu,Zn-SOD) at 4°C. The tissues were then incubated with enhanced labelled polymer peroxidase (Dako K1491). The reaction product of antigen-antibody was visualized using diamino benzidine (DAB). The tissue sections were then counterstained with haematoxylin, dehydrated with series of alcohol, and cleared with xylol. Finally, the sections were mounted with entelan. As control of staining, tissue sections were incubated with PBS instead of Cu,Zn-SOD antibody. The tissue sections of control staining showed negative reaction with minimal background staining.

Observation and Data Analysis. The immunoreaction products of the SOD were observed by using a light microscope. The distribution and frequency of positive reaction product on the liver tissues of control group were compared qualitatively and quantitatively to that of the diabetic groups. The qualitatively observation of Cu,Zn-SOD reaction product was done to the cytoplasm of hepatocytes, as well as quantitatively in the nucleus of the cells. The reaction product of Cu,Zn-SOD in the nucleus was graded based on the colour intensity of reaction product, from brown (positive) to blue (negative) colour. There are four grades of reaction product; (a) strong positive (+++), strong brown colour, showed high concentration of the enzyme, (b) moderate positive (++), light brown colour, (c) weak positive (+/-), mixed light brown and blue colour, and (d) negative reaction product, blue colour. The hepatocytes in processing to death showed negative reaction product (blue colour) of Cu,Zn-SOD in their nucleus. The cells in different degree of reaction product to Cu,Zn-SOD in the control and diabetes groups were counted per view of 400 magnification. There are five views observation per each sample. The number of hepatocytes in different degree in the control group was compared statistically (Anova) to that of diabetes groups.

RESULTS

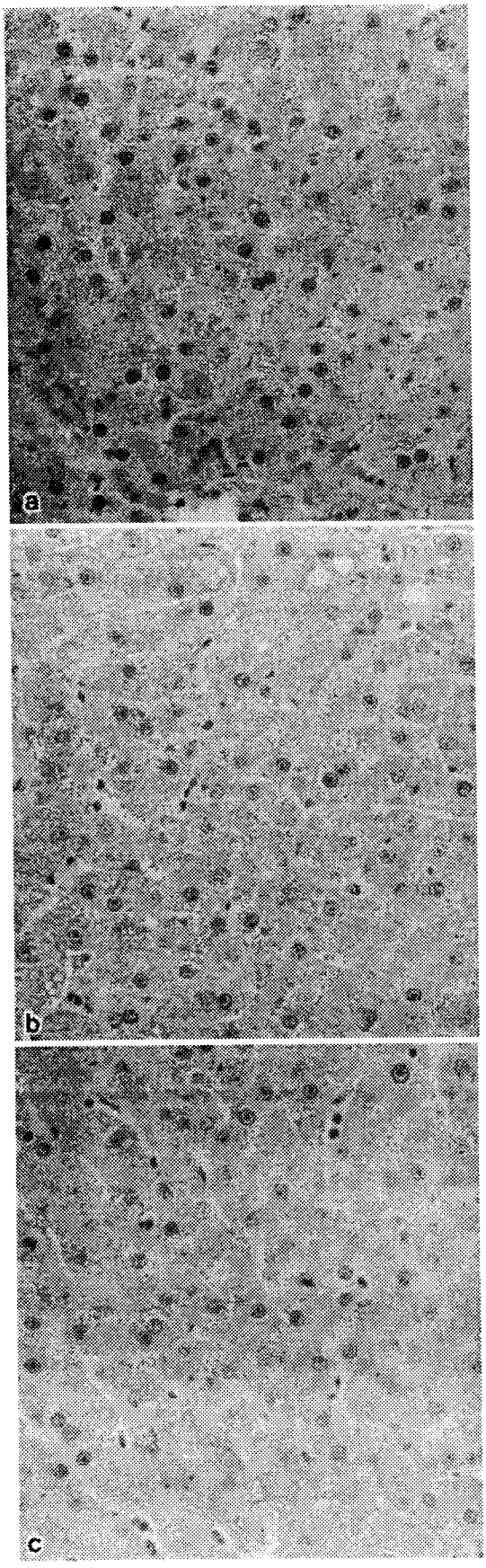
The immunohistochemical localization of Cu,Zn-SOD showed positive reaction in the liver of *Macaca fascicularis*. The enzyme was localized both in the nuclear and cytoplasm of hepatocytes, while the Kupfer cells, in the sinusoidal spaces, are negative to the enzyme. The reaction product of the enzyme in the liver tissues of diabetic groups qualitatively showed differences from those of the control group, especially in the cytoplasm of hepatocytes. The Cu,Zn-SOD content showed a decrease in the diabetic groups when compared to the control group (Figure 1). The decrease of the intracellular antioxidant was more clearly shown in the IDDM group than in the NIDDM group.

The hepatocytes in processing to death are negative to the Cu,Zn-SOD content. The negative reaction product was more clearly shown in their nucleus than in the cytoplasm. The nuclei of negative cells were graded in relation to their relative negative reaction product. The number of hepatocytes in different degrees of reaction products to the enzyme, as viewed in 400x magnification, in the male *Macaca fascicularis* is quantitatively shown in Table 1. The intracellular antioxidant in the liver of diabetes mellitus groups quantitatively showed a decrease compared to the control group. The decreasing of the enzyme was shown by the decrease in the number of strongly positive-cells (+++), and the increase in the number of moderate positive-cells (++) and negative-cells (-) in the liver tissues of diabetes mellitus groups compared to the control group. It was more remarkable in the IDDM group than in the NIDDM group (Table 1).

The percent number of hepatocytes, in every degree of reaction product of Cu,Zn-SOD, to the total hepatocytes in the liver tissues, when viewed at 400 magnification, also showed the decrease of the enzyme in the diabetes mellitus groups when compared to the control group. The decrease shown by the decrease in percentage of positive-cells, and increase in percentage of negative-cells to the total hepatocytes in the liver tissues of diabetes mellitus groups as compared to the control group are shown in Figure 2.

Histopathological evidence also occurred in the diabetes mellitus groups. It was more remarkable in the IDDM group than in the NIDDM group. The evidence showed a degenera-

tive process; picnosis in the nuclei of hepatocytes, increase of polymorphonuclear (inflammatory) cells, and remarkable fat degeneration in the IDDM group (Figure 1d).



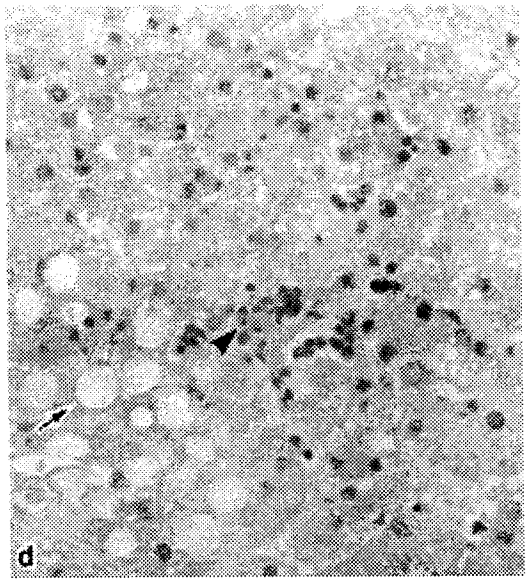


Figure 1. The micrographs of Cu,Zn-SOD localization in the liver of *Macaca fascicularis*: a. control group, b. NIDDM group, c. IDDM group, and d. fat degeneration (arrow) and inflammatory cells (arrowhead) of IDDM group. The enzyme in the diabetes groups shown decreased qualitatively in the cytoplasm, as well as quantitatively in the nucleus of hepatocytes. It was more dramatic in the IDDM group than in the NIDDM group.

Table 1. The number of hepatocytes in different degrees of reaction products to Cu,Zn-SOD in male diabetic Macaques (*Macaca fascicularis*), at 400 times magnification

Group	Number of hepatocytes in different degree of reaction product to Cu,Zn-SOD			
	+++	++	+/-	-
Control	65.67c	27.00a	37.67a	6.67a
NIDDM	30.33b	41.33b	36.67a	21.00b
IDDM	17.33a	48.33b	40.67a	36.33c

Different superscript letters at the same coloum are significantly different ($P < 0.05$)

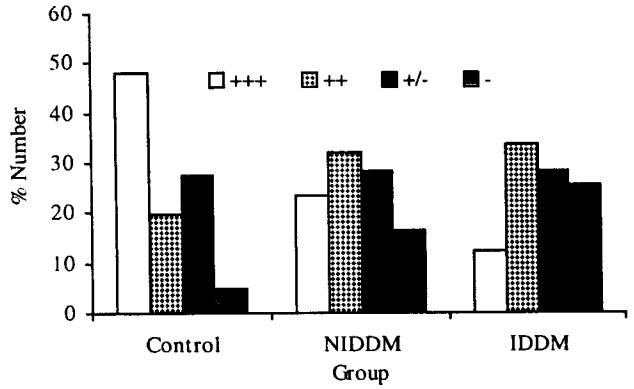


Figure 2. The percent number of hepatocytes in different degree of reaction product to Cu,Zn-SOD (+++: strong positive, ++: moderate positive, +/-: weak positive, and -: negative) in the liver of Macaques (*Macaca fascicularis*), at 400 times magnification. NIDDM: non-insulin-dependent diabetes mellitus. IDDM: insulin-dependent diabetes mellitus.

DISCUSSION

It was reported that numerous alterations in hepatic ultrastructure and metabolism occur during diabetes and starvation (Thomas *et al.* 1989). The streptozocin-induced diabetes mellitus showed changes in carbohydrate metabolism, and resulting hyperglycemic condition (Litwak *et al.* 1998). The alterations were caused by the absence or decrease of insulin production, as well as decrease of insulin function. In this condition, release of fatty acid from adipose tissue into the blood stream is stimulated. The free fatty acids are incorporated into organs, oxidized in the cells of these organs and utilized as energy sources instead of glucose. As a consequence, the rate of fatty acid β-oxidation is increased (Thomas *et al.* 1989).

Nilsson *et al.* (1987) and Hawkins *et al.* (1987) also reported that under normal conditions, the peroxisomal β-oxidation is only a minor pathway for fatty acid oxidation. However, during diabetes and starvation, this pathway is enhanced.

It was also reported that high levels of free fatty acids or their metabolites, which are known to accumulate in the liver, act as endogenous peroxisome proliferators (Thomas *et al.* 1989). Peroxisomes play an important role in certain oxidations (Zaar 1992). Langseth (1995) reported that oxidation and reduction in peroxisome and mitochondria resulted in the release of oxygen-free radicals.

Orellana *et al.* (1992) reported that starvation and diabetes increase the cytochrome P-450 that oxidizes fatty acids, as well as peroxisomal β-oxidation, in the rat liver and kidney. The peroxisomal and cytochrome P-450 oxidations resulted in reactive oxygen species, superoxide anions (O_2^-) by cytochrome P-450 oxidation, and hydrogen peroxide (H_2O_2) by peroxisomal β-oxidation (Mates *et al.* 1999). The cytochrome P-450 oxidation was reported to occurred in the endoplasmic reticulum (Orellana *et al.* 1992) and peroxisomes (Dhaunsi *et al.* 1992). Thus, the diabetes condition may produce more reactive oxygen species. The condition with highly reactive oxygen species, oxidative stress, can readily react with various biological macromolecules of cells resulting in protein destruction. The lesions in turn lead to degenerative processes (Forrest *et al.* 1994), as shown in the liver tissues of diabetes mellitus groups in this study. Therefore, in order to defend tissue damage from the oxidant, large amount of intracellular antioxidant including Cu,Zn-SOD were needed to catalyses the highly reactive oxygen. Subsequently, the Cu,Zn-SOD content in the liver tissues decreased in the experimental diabetes mellitus Macaque (*Macaca fascicularis*) groups compared to the control group.

The decrease of the Cu,Zn-SOD is more remarkable in the IDDM group than in the NIDDM group. In the IDDM group, insulin is absolutely absent. The condition resulting in alteration of the glucose metabolism, and oxygen-free radical as a side effect from highly fatty acid oxidation may significantly occur in the IDDM group when compared to the NIDDM group. The blood glucose level in the IDDM group (>350 mg%) is also higher than that of the NIDDM group (120-350 mg%). It was reported that high level of blood glucose resulted stress oxidative condition with high levels of oxygen-free radical (Soeatmadji 2001). Both these conditions may cause the decrease of Cu,Zn-SOD in the IDDM group in a more dramatic way than in the NIDDM group.

These results showed that Cu,Zn-SOD decreased in the liver tissues of the experimental diabetes mellitus Macaques (*Macaca fascicularis*) when compared to the control group. The decrease was more remarkable in the IDDM group than in the NIDDM group. The decrease of the enzyme may be caused by an increase of oxygen-free radicals resulting from the condition. These results account for the involvement of intracellular antioxidant Cu,Zn-SOD, in the antioxidant defense system of the male *Macaca fascicularis* under diabetes mellitus conditions, in order to defend tissue damage from the oxygen-free radical. Thus, further efforts are necessary to fully elucidate the importance of antioxidant enzymes in the therapy of several human disease conditions, especially of diabetes mellitus.

ACKNOWLEDGMENT

We are deeply grateful to Koichi Mamba of Department of Veterinary Anatomy, Yamaguchi University, Yamaguchi - Japan, for providing Dako envision polymer peroxidase.

REFERENCES

Aguirre F, Martin I, Grinspon D, Ruiz M, Hager A, De Paoli T, Ihlo J, Farach HA, Poole CPJr. 1998. Oxidative damage, plasma antioxidant capacity, and glucemic control in elderly NIDDM patients. *Free Radic Biol Med* 24:580-585.

Ames BN, Shigenaga MK. 1992. DNA damage by endogenous oxidants and mitogenesis as causes of aging and cancer. In: Scandalios (ed). *Molecular Biology of Free Radical Scavenging Systems*. New York: Cold Spring Harbor Laboratory. p 1-22.

Cerami A, Vlassara H, Brownlee M. 1988. Role of advanced glycosylation products in complications of diabetes. *Diabetes Care* 11:73-79.

Dhaunsi GS, Gulati S, Avtar KS, John KO, Asayama K, Inderjit S. 1992. Demonstration of Cu,Zn-superoxide dismutase in the rat liver peroxisomes. *J Biol Chem* 267:6870-6873.

Dobashi K, Asayama K, Kato K, Kobayashi M, Kawaoi A. 1989. Immuhistochemical localization and quantitative analysis of superoxide dismutase in rat tissue. *Acta Histochem Cytochem* 22:351-365.

Forrest VJ, Kang YH, Mc Clain DE, Robinson DH, Ramakrishnan N. 1994. Oxidative stress apoptosis prevented by trolox. *Free Rad Biol Med* 16:675-683.

Freisleben HJF. 2001. Free radicals and ROS in biological systems. In: *Radikal Bebas dan Antioksidan dalam Kesehatan*. Jakarta: Bagian Biokimia, Fakultas Kedokteran, Universitas Indonesia. p 1-21.

Hawkins J, Jones WE, Bonner FW, Gibson GG. 1987. The effect of peroxisome proliferators on microsomal, peroxisomal, and mitochondrial enzyme activities in the liver and kidney. *Drug Metab Rev* 18:441-451.

Howard CF. 1982. Nonhuman primates as models for the study of human diabetes mellitus. *Diabetes* 31:37-42.

Howard CF, Yasuda M. 1990. Diabetes mellitus in nonhuman primates recent reseach advances and current husbandry practices. *J Med Primatol* 19:609-625.

Kakkar R, Mantha SV, Radhi J, Prasad K, Kalra J. 1998. Increased oxidative stress in rat liver and pancreas during progression of streptozotocin-induced diabetes. *Clin Sci (Lond)* 94:623-632.

Langseth L. 1995. *Oxidants, Antioxidants and Disease Prevention*. London: ILSI.