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Immunohistochemical Study of Oxygen-Free Radical Scavenger-Copper, Zinc-Superoxide Dismutase (Cu,Zn-SOD) in the Rats Liver under Stress Condition

Deteksi Secara Imunohistokimia Oxygen-Free Radical Scavenger-Copper, Zinc-Superoxide Dismutase (Cu,Zn-SOD) pada Hati Tikus di Bawah Kondisi Stress

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Abstrak

Copper,zinc-superoxide dismutase *(Cu,Zn-SOD) yang merupakan salah satu* oxygen-free radical scavenger *telah dideteksi secara imunohistokimia pada hati tikus di bawah kondisi stres. Sejumlah empat puluh lima ekor tikus jantan galur Wistar telah digunakan pada penelitian ini. Hewan percobaan tersebut dikelompokkan menjadi tiga kelompok, yaitu : (1) kelompok kontrol, (2) kelompok dengan perlakuan stres selama 3 hari, dan (3) kelompok dengan perlakuan stres selama 5 hari. Stres yang diberikan adalah puasa, dengan pemberian air minum secara* ad libitum. *Stres yang diberikan menimbulkan keadaan histopatologis, peradangan dan nekrosis pada jaringan hati kelompok perlakuan tersebut. Secara imunohistokimia menunjukkan adanya penurunan kandungan Cu,Zn-SOD pada jaringan hati kelompok perlakuan dibandingkan pada kelompok kontrol. Penurunan kandungan Cu,Zn-SOD tersebut lebih hebat pada kelompok perlakuan selama 5 hari dibandingkan dengan kelompok perlakuan selama 3 hari. Hasil penelitian ini menunjukkan bahwa kondisi stres kemungkinan dapat meningkatkan terbentuknya* oxygen-free radical *yang kemudian merusak jaringan hati dan menurunkan kandungan Cu,Zn-SOD.*

Kata kunci : imunohistokimia, hati, stres, superoxide dismutase *(SOD)*

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Introduction

Superoxide dismutase (SOD) is one of oxygen-free radical scavenger, antioxidant enzyme. The enzymes play a significant role in direct protection of cells against oxidatives stress and indirectly maintain a balance among the various toxic oxygen species (Touati, 1992). The protection can be done by several ways such as preventing, stopping or decreasing of oxidations (Schuler, 1990), as well as 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 defence against the most reactive superoxide anion radical generated intra-cellularly. The distribution of SOD in the rat tissues was reported immunohistochemically by Dobashi *et al*.(1989), Kawada *et al*.(1996), Munim *et al.* (1992) and immunocyto-chemically 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. These highly reactive oxygens can readily react with various biological

macromolecules such as DNA, proteins, lipids, and caused protein destruction. The lesions in turn lead to various diseases and degenerative processes such as aging and carcinogenesis in human and animals (Ames and Shigenaga, 1992).

Previous research on renal peroxisomes under fasting stress condition (Wresdiyati and Makita, 1995) revealed several alterations such as remarkable increase on their number. Peroxisomes have an important role in certain cellular oxidations, such as lipid β-oxidation, D-amino acid oxidation, D-aspartat oxidation, L-α-hydroxyacid oxidation, urate oxidation, etc. (Zaar, 1992). Free radical endogenously generated from reductions and oxidations reactions, which naturally take place in mitochondria and peroxisome, or detoxification of xenobiotic, etc. (Langseth, 1995). As a consequence, fasting stress condition, which increased the number of peroxisome, may also induce some oxidations and produce active oxygen species.

This study was designed to reveal the effect of stress condition of the Cu,Zn-SOD in the rats liver immunohistochemically. The observation was done qualitatively in the cytoplasm of hepatocytes, as well as quantitatively in the nucleus of the cells, which was expected to give different degree of reaction products to Cu,Zn-SOD.

Materials and Methods

*Treatment of Animals***:** A total of 45 male Wistar rats (230 – 250 g) were used for this experiment. They were divided into three groups; control, three-days fasting, and five-days fasting stress group. All animals were placed in appropriate cages and stabilized with the housing condition for a week. The control group was fed with a rat commercial diet and drinking water *ad libitum*, while the fasting groups were given drinking water only for 3 and 5 days.

*Tissue Preparation***:** After the treatment, all animals were decapitated, and pieces of tissues from liver were fixed with 4 % paraformaldehyde in phosphate buffer saline (PBS). Tissues were dehydrated through a graded ethanol series, embedded in paraffin and cut into 4 μm-thick sections and subjected to histopathological and immunohistochemical studies.

*Histopathology***:** The tissue sections were stained with haematoxylin and eosin method.

Immunohistochemistry: SOD was localized immunohistochemically as describe previously (Dobashi *et al.*, 1989) with a modification. The tissue sections were washed for 15 min with three changes of 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. After 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: Haematoxylin and eosin stained tissue sections were observed under a light microscope. The cell condition and some histopathological signs of the tissues from the treatment groups were compared to that of the control group. Observation to the liver tissues was done to the condition of hepatocytes and the interstitial cells area. The polymorphonuclear cells in the interstitial cells were counted per view of 400 magnification. There are five views observation per each sample. The number of polymorphonuclear cells in the tissues of control group was statistically (t-test) compared to that of fasted groups.

The immunoreaction products of the SOD were also observed by using a light microscope. The distribution and frequency of positive reaction product on the tissues of

control group were compared qualitatively and quantitatively to that of the treatment groups. The qualitatively observation of Cu,Zn-SOD reaction product was done to the cytoplasm of hepatocytes, while quantitatively observation of the enzyme was done to 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 hepatocyte in processing to death showed negative reaction product (blue colour) of Cu,Zn-SOD in their nucleus. The hepatocyte in different degree of reaction product to Cu,Zn-SOD in their nucleus in both control and fasted groups were counted per view of 400 magnification. There are five views observation per each sample. The number of the cells in different degree in the control group was compared statistically (Anova) to that of fasted groups.

Results and Discussion

Histopathology: Histopathological evidence was observed in the liver tissues stained by haematoxylin and eosin (HE). The hepatocytes were arranged radially to the central vein with the presence of Kupffer cells in their sinusoidal spaces. Both in the three days and five days fasted stress groups showed alterations of their liver tissues, some of hepatocytes showed pycnosis in their nuclei. The number of hepatocytes in the five days fasted group bigger than that of the three days fasted group. The number of polymorphonuclear cell was remarkable increased in their sinusoidal spaces of fasted groups than that of the control group. The increasing of the number of polymorphonuclear cells is showed in Table 1. These data showed that fasting stress resulted inflammation (Ringler, 1996) in the liver tissues of rats.

Previous research on renal peroxisomes of Japanese monkeys under fasting stress condition reported several alterations such as remarkable increased on their number (Wresdiyati and Makita, 1995). Peroxisomes play an important role in certain oxidations. Langseth (1995). Reported that oxidations and reductions in peroxisomes and mitochondria resulting oxygen-free radical. As a consequence, fasting stress condition, which increased the number of peroxisomes, may increase peroxisomes oxidations.

Immunohistochemistry: Cu,Zn-SOD was immunohistochemically localized in the nuclei and cytoplasm of the hepatocytes. The positive reaction product of the enzyme in the liver tissues of the fasted groups qualitatively showed significantly different from control group (Figure 1). The enzyme activity gradually decreased in the fasted groups than control group. The decreasing of the enzyme both in cytoplasm and nuclei was related to the length of fasting stress. It was more dramatic in the five days than that of three days fasted group.

The decreasing of the Cu,Zn-SOD was also showed by the quantitatively in the nuclei of hepatocytes, which give different degree of reaction product to Cu,Zn-SOD (Table 2). The hepatocytes in the process to death are negative to the Cu,Zn-SOD content. The negative reaction products were more clearly showed the nuclei than cytoplasm (Figure 1). The decreasing of the enzyme showed by the increase number of hepatocytes which give negative reaction product and moderate positive-cells, as well as by decreasing number of strong positive-cells (Table 2). These results related to the length of fasting stress, the decreasing of the enzyme more dramatic in the five days than the three days fasted stress group.

The percent number of cells, in every degree of reaction product of Cu,Zn-SOD, to the total hepatocytes was also showed the decrease of the enzyme more in the fasted groups than that of the control group (Figure 2).

In the fasted animals, the blood glucose level decreased and released of fatty acids from adipose tissue into the blood stream was

stimulated. The free fatty acids were incorporated into organs, oxidized in the cells of these organs and utilized as energy sources instead of glucose (Ishii *et al.*, 1980). Gaal (1993) also reported that fasting stress produce in immediate decrease in blood glucose accompanied by an increase of free fatty acid, total lipid, total cholesterol and urea in plasma.

The peroxisomal oxidizing system plays an important role in the oxidation of fatty acid originating from the adipose tissue. The activity of peroxisomal oxidation was showed increase more rapidly and markedly than that of mitochondrial oxidation during fasting stress condition. The fasting stress was also reported increased the cytochrome P-450 that oxidize fatty acid, as well as peroxisomal β-oxidation, in the rat liver and kidney (Orellana *et al*., 1992). The peroxisomal and cytochrome P-450 oxidations resulting 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 occurred in the endoplasmic reticulum (Orellana *et al*., 1992) and peroxisomes (Dhaunsi *et al*., 1992). The increase number of peroxisomes under fasting stress (Wresdiyati and Makita, 1995) may increase the number of reactive oxygen species, oxygen-free radical. Therefore, in order to defense the tissue damage from the oxidant, large amount of Cu,Zn–SOD were needed to catalyses the dismutation of the highly reactive superoxide anions to O_2 and to the less reactive species H_2O_2. Subsequently, the Cu,Zn–SOD content in the liver tissues decreased in fasted groups than that of control group. The gradual decreased of the enzyme related to the length of fasting stress. It suggested that reactive oxygen species more markedly increase in the longer time of fasting stress.

Conclusion

These results showed that fasting stress condition may increase reactive oxygen species, therefore it decreased the content of intracellular antioxidant Cu,Zn–SOD in the liver tissues of rats. It might account for the involvement of intracellular antioxidant Cu,Zn-SOD, in the antioxidant defense system of male Wistar rat liver tissues under fasting stress condition, in order to protect tissue damage from the oxygen-free radical.

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Table 1. *The number of polymorphonuclear (inflammatory) cells, in the sinusoidal spaces of liver tissues in the male Wistar rats*

Group	Number of poplymorphonuclear cells (per view of 400 magnification)
Control	9.80 ± 0.5
3 days Fasted	28.65 ± 2.4**
5 days Fasted	46.63 ± 7.6**

** Significantly different to the control group, P<0.001 (t-test)

Table 2. *The number of hepatocytes in different degree of reaction products to Cu,Zn-SOD in the male Wistar rats under stress condition, per view of 400 magnification*

Group	Number of hepatocytes in different degree of reaction product to Cu,Zn-SOD			
	+++	++	+/-	-
Control	87.67±9.76[c]	8.67±0.98 [a]	0.67±0.02 [a]	7.00±0.82 [a]
3 days Fasted	7.00±1.42 [b]	78.67±10.30[c]	29.00±8.97 [b]	56.67±18.01[b]
5 days Fasted	2.00±0.51[a]	33.00±7.42 [b]	37.5±9.26 [b]	74.5±20.04 [b]

Different superscript letters are significantly different (P< 0.05)

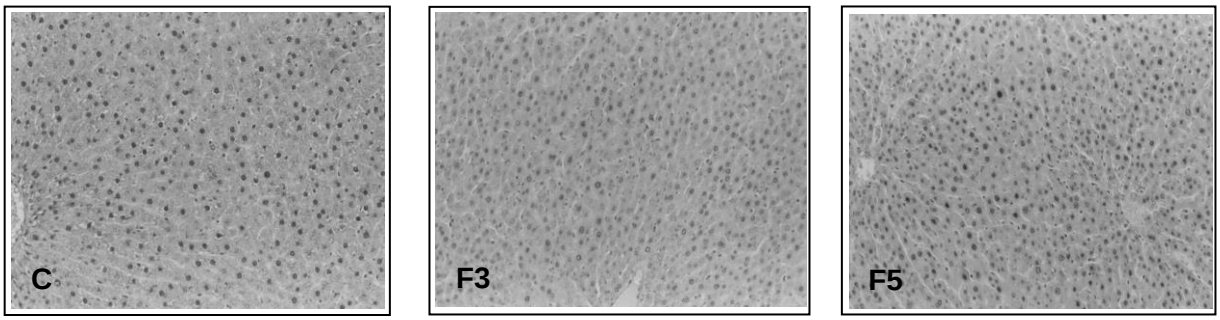

Figure 1. *The micrographs of Cu,Zn-SOD localization in the liver of male Wistar rats. C : control group, F3 : 3 days fasted group, F5 : 5 days fasted group. The enzyme in the fasted groups showed qualitatively decreased in the cytoplasm and nuclei of the hepatocytes than that of control group. The decrease of the antioxidant more dramatic in the 5 days fasted group than that of 3 days fasted group. Magnification : 20x10*

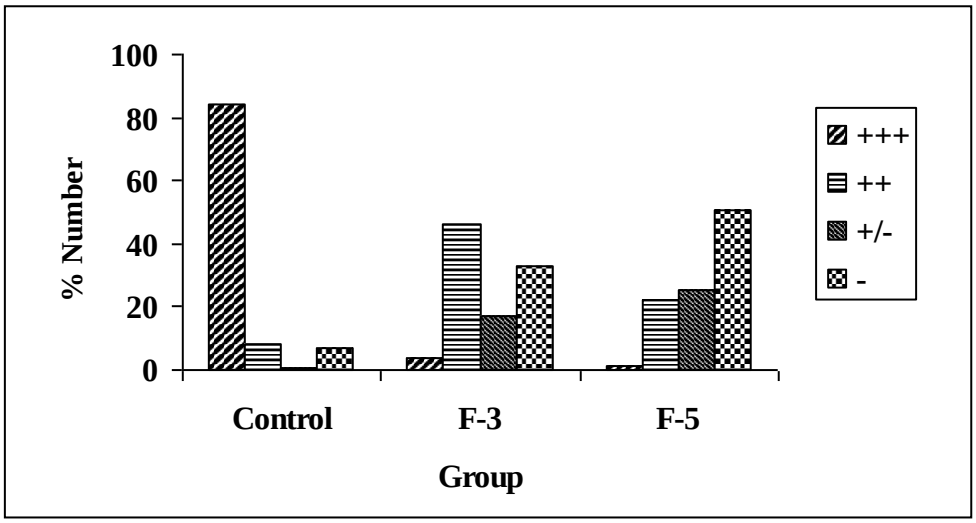

Figure 2. *The percent number of hepatocytes in different degree of reaction product to Cu,Zn-SOD (+++/strong positive, ++/ moderate positive, +/- /weak positive, and -/negative) in the male Wistar rats under stress condition, per view of 400 magnification. F3 : 3 days fasted group, F5 : 5 days fasted group.*