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Superoxide Dismutase Activity and Lipid Peroxidation in the Liver of Guinea Pig Infected with *Leptospira interrogans*

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Superoxide dismutase (SOD) activity and the degree of lipid peroxidation were studied over a two week period in guinea pigs infected with *Leptospira interrogans* derived from wild mice. The total SOD activity in infected host liver increased by four-fold two days after infection; this was followed by a 20% decrease resulting in levels comparable to normal, uninfected liver. During the period of decreasing SOD activity after day two, the levels of TBA-reactive material (TBARS) are increased by three-fold in infected guinea pig, liver, compared to uninfected liver. The results indicate that SOD attenuates intracellular superoxide-mediated toxic effects in guinea pigs infected with *L. interrogans*. In addition, electron microscopy structure demonstrates correlated pathogenic shrinkage of mitochondrial and Kupffer cell structures.

Keywords: Leptospira, Leptospirosis, Guinea pig, Superoxide dismutase, Lipid peroxidation, Malondialdehyde

INTRODUCTION

Leptospirosis is a widely distributed infectious disease of known etiology; *L. interrogans* is a common causative agent in Korea as well as in other areas of the world.[1,2,3] Leptospirosis is characterized by hemorrage in the lung[4] and necrosis of the liver[5] and kidney.[6] This pathology is associated with glycolipoprotein[7] and lipopolysaccharide[8] alterations, and therefore may be due to lipid peroxidation in cell membranes via reactive oxygen species. Accompanying hemolysis is caused by the oxidation of hemoglobin[7] and by the lipid peroxidation of erythrocytes.[9] This effect could result from a decrease of antioxidants as a possible mechanism of leptospirosis.

Data describing SOD deficiency suggest that intracellular oxygen radicals and the final lipid peroxidation product malondialdehyde are increased.[10,11] Thus, we surmised that one of the key antioxidants involved in this protection might be SOD, which catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen. The mammalian CuZnSOD is characteristically found in the cytosol, whereas MnSOD

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is found in mitochondrial matrix.[12] Alterations of enzymes such as catalase and glutathione peroxidase in the host animals infected with *Leptospira species* have not been described, but may be critical in mediating the cellular response to superoxide radicals.

Recently, experimental leptospirosis has been studied in various species of monkeys with acute illness including jaundice, hemorrhagic syndrome[13] and degradation of membrane sphingomyelin.[14] This leptospirosis was found to be severe in respiratory, renal,[15] and hepatic organs.[16] These species were shown to be highly susceptible to *L. interrogans* such as copenhageni and icterohaemorrhagie as compared to guinea pigs and Mongolian gerbil.[17] In this study, therefore, we assessed the changes in SOD activity and the level of lipid peroxidation in guinea pig liver infected with *L. interrogans*. We also examined the pathogenic structure of organelles in leptospiral liver by electron microscopy.

MATERIALS AND METHODS

Isolation of L. interrogans and Liver of Guinea Pig

L. interrogans was isolated from kidney tissue of wild mice collected from Chollanamdo in Korea. Kidney tissue was sliced and immersed in 0.02 M phosphate buffer (pH 7.4). One drop diluted 1:10 in the same buffer was spread on the Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco) containing 10% Bacto-Leptospira enrichment and 0.15% Vegetable gelatin. The cells were cultured at 37°C for six days, and then identified by dark field microscopy (×450). Cells (8.9×10^9 cells/ml) diluted 1:10 in 1 ml phosphate buffer (pH 7.4) were injected intraperitoneally into each guinea pig (200 ± 10 g). Guinea pigs were housed under constant environmental conditions (temperature: 22 ± 1°C; relative humidity: 60 ± 5%; circadian rhythm: 12 hr light and 12 hr dark). They were grown with standard nutritional food supplemented with water ad libitum. About 3–5 guinea pigs were examined every three days for two weeks. Guinea pigs were killed by decapitation, and the liver was removed and rinsed with ice cold saline. Pathogenic leptospirosis was stained with hematoxylin eosin and examined by light microscopy.

Electron Microscopic Study

The liver specimens (1 mm^3) were sliced and placed in fixative, 1% OsO_4 buffer with 0.1 M collidine, 2.5% glutaraldehyde buffered with 0.1 M cacodylate followed by postfixation in OsO_4. The tissues were dehydrated with increasing concentrations of ethanol, then placed in propylene oxide and embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate, and examined using an electron microcope (JEM 100 CX-II).

Crude Extracts and Enzymatic Assays

The liver samples (2 g) were homogenized using a ultra-turrax T-25 homogenizer (Germany) in 3 ml of 0.05 M phosphate buffer with 0.1 mM EDTA (pH 7.8) at 0°C, and the homogenates were stored at –80°C if necessary. Homogenized liver sample (4 ml) was treated with Triton-X 100 (0.1%, v/v) and centrifuged at 15,000 g for 5 min, thereby removing nuclear fractions. Cytoplasmic and mitochondrial enzyme fractions could not be isolated simultaneously, therefore these fractions were isolated separately and, for simplicity, later combined and run on the gels as described below. To obtain total enzyme activity, the supernatant was recentrifuged at 60,000 g for 30 min at 4°C. The supernatant, which corresponded to the cytosolic fraction, was collected, immediately frozen, and stored at –80°C until assay. The pellets were resuspended in fresh 0.05 M phosphate buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM EDTA and then centrifuged at 60,000 g for 60 min at 4°C. The second supernatant corresponded to the mitochondrial fraction. The details were followed as described elsewhere.[11,18] Protein

concentration was determined by the method of Lowry *et al.*[19] SOD activity was quantitated by the xanthine oxidase-cytochrome c method as described by McCord and Fridovich.[20] Enzyme activity in 12% non-denaturing polyacrylamide gels was visualized by using the method of Beauchamp and Fridovich.[21] Then, 40 μg total fraction (cytoplasmic : mitochondrial fraction = 20 : 20 μg) was loaded on the same lane of the gels. Activities of CuZnSOD and MnSOD were compared with commercial bovine CuZnSOD (Sigma). The activities of CuZnSOD and MnSOD were determined with and without inclusion of 1 mM cyanide in the incubation mixture by using the method of Ledig *et al.*[22]

Lipid peroxidation was determined by the formation of thiobarbituric acid-reactive substances in a 10 minute period and expressed as a concentration of malondialdehyde. The reaction mixture containing 8% sodium dodecyl sulfate, 20% acetic acid (pH 3.5) and 0.8% thiobarbituric acid was heated at 90°C for 60 min. After cooling, a n-butanol and pyridine mixture (15:1, v/v) was added, and then shaken vigorously and centrifuged at 1,000 g for 10 min. The absorbance of the supernatant was measured at 532 nm at room temperature. 1,1,3,3-Tetramethoxypropane was used as an external standard, and the levels of lipid peroxides were expressed as nmol of MDA produced. The details were followed as described by Okawa et al.[23]

RESULTS

Pathogenic Changes

Guinea pigs infected with *L. interrogans* are highly susceptible to leptospirosis, demonstrating with acute illness and hemorrhagic syndrome, affecting of all the infected animals by 13 days after inoculation. Electron micrographs of hepatocytes of guinea pig infected with *L. interrogans* show that Kupffer cells were decreased, and mitochondria appeared to be damaged and severely shrunken compared with controls 6 days after injection (Fig. 1).

SOD Activities

Non-denaturing polyacrylamide gel electrophoresis showed that the total SOD activity of the liver of guinea pig infected with *L. interrogans* was approximately four fold higher than that of controls 2 days after infection. Data shown in Figure 2 and Figure 3 indicate that at day two after infection both CuZnSOD and MnSOD in livers of guinea pigs were increased 4-fold as compared to control animals. After day 2, the activities of both CuZnSOD and MnSOD declined and reached a level similar to the control (day 1) (see Fig. 2 and Fig. 3). The rate of decline in CuZnSOD was not significantly different from that of MnSOD.

Lipid Peroxidation

Infection with *L. interrogans* caused a marked increase in liver TBARS (expressed by MDA concentration) with a maximal value reached on the 11th day after injection (Fig. 2). This level was approximately 3-fold over that of controls. In order to correlate the changes in the SOD with those of the liver lipid peroxidation after *L. interrogans* infection, the percentage of MDA values and SOD activities were plotted against day after injection. A decrease in SOD activity is correlated with increased TBARS of the liver in guinea pigs infected with *L. interrogans*, and thus may lead to critical damage to the other organs such as lung and kidney.

DISCUSSION

This study describes the SOD activity and the levels of TBARS, as an index of lipid peroxidation, in livers of guinea pigs infected with *L. interrogans*. Pathogenesis of acute leptospires has been known to form glycolipoprotein (GLP) cytotoxin which may be part of the leptospiral cells.[7] The leptospires can lead to secondary ischemic damage to organs such as liver, kidneys, and adrenals.[24] All lines of evidence point to an effect of toxin on host

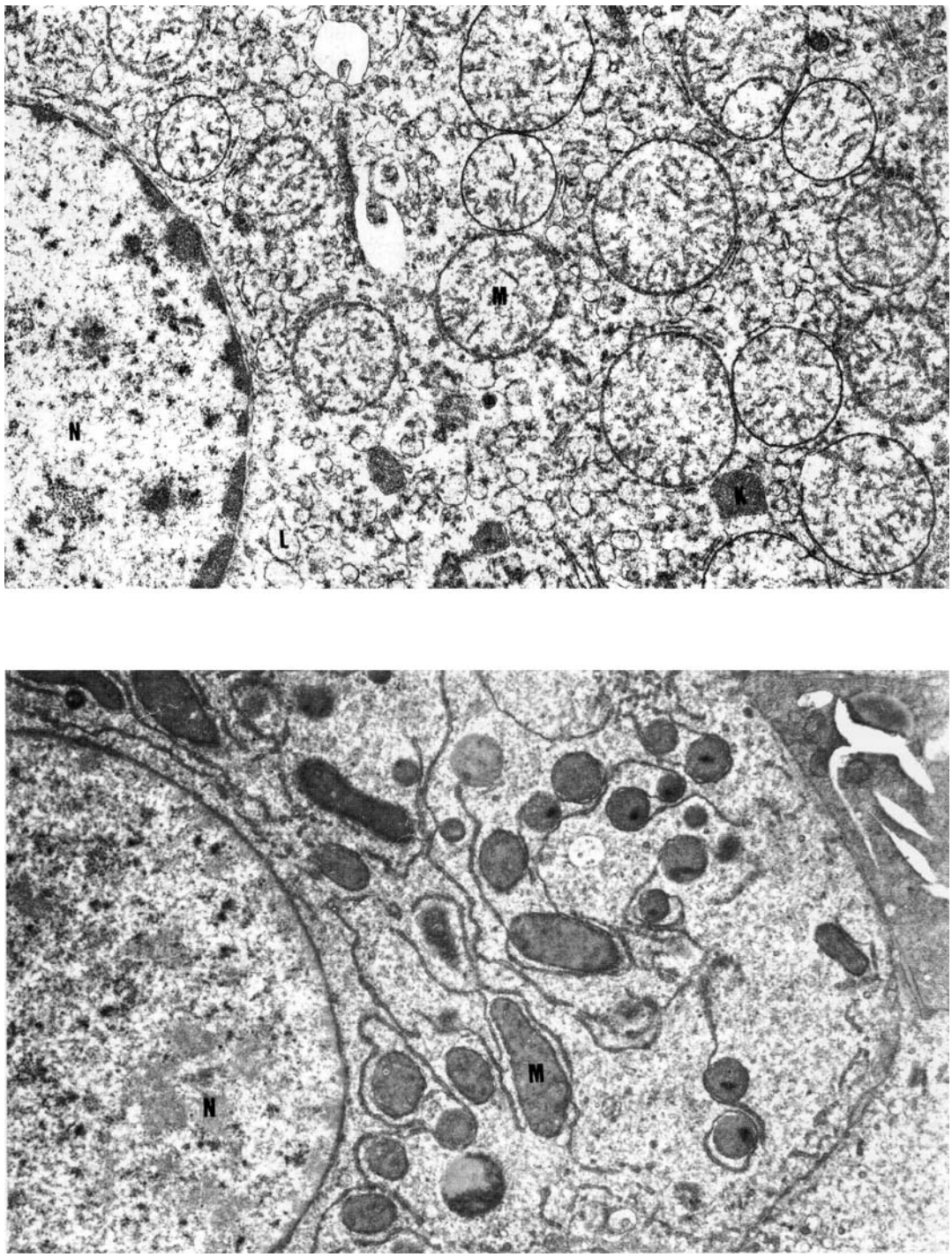

FIGURE 1 Electron microscopic observation of hepatocyte on sixth day of guinea pig infected *L. interrogans* (× 53,000). N; nucleus, M; mitochondria, K; Kupffer cell, L; lysosome (a); control, (b); Infection.

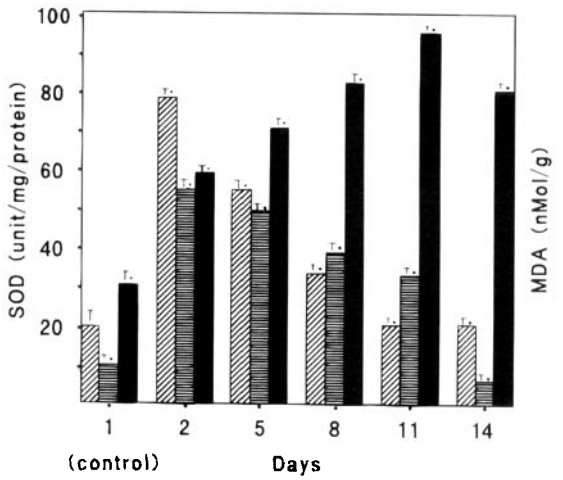

FIGURE 2 SOD activity in the liver of guinea pigs infected with *L. interrogans*. ❑ CuZnSOD (unit/mg protein), ❑ MnSOD (unit/mg protein). ■ TBARS (as nmol MDA/g). •, $p < 0.05$ against controls.

cell membranes. Membrane permeability is disturbed by the intercalating of leptospiral lipids or of phospholipid which leads to GLP toxicity.[7,25,26] Therefore, it has been reasonably expected that oxygen radicals should be produced and involved in this form of toxic damage. Results of the present study show that SOD in liver cells was significantly higher ($p < 0.01$) in the infected group 2 days after infection. However, thereafter this activity gradually decreased and showed an inverse relationship with the level of MDA. These findings suggest that *L. interrogans* infection led to increased SOD activity and increased lipid peroxidation. Such lipid peroxidation was significant, but was slightly inhibited during the first 2 days of infection. However, peroxidation increased 2 days after infection which was correlated with a decrease in SOD activity in the liver of infected guinea pigs. This suggests that the rise in SOD may function temporarily to decrease lipid peroxidation and this function may delay the cellular damage. It has been suggested that the reduced activity of SOD observed in the mouse liver may account for the elevated level of oxygen radicals and hence enhance lipid peroxidation in the liver of γ-irradiated mice.[27] Mitochondria are considered to be the main physiological generator of oxygen radicals in the liver. Therefore, mitochondrial damage will cause breaks in the electron transport chains and thereby damage respiratory function. The lesions observed in infected guinea pig liver are consistent with these observations. It is possible to suggest tentatively that initial increase in SOD activity (2 days post-infection) may function as a protective antioxidant and attenuate lipid peroxidation. Further studies to correlate the relationship between *L. interrogans* infection and other oxidative damages (e.g., DNA

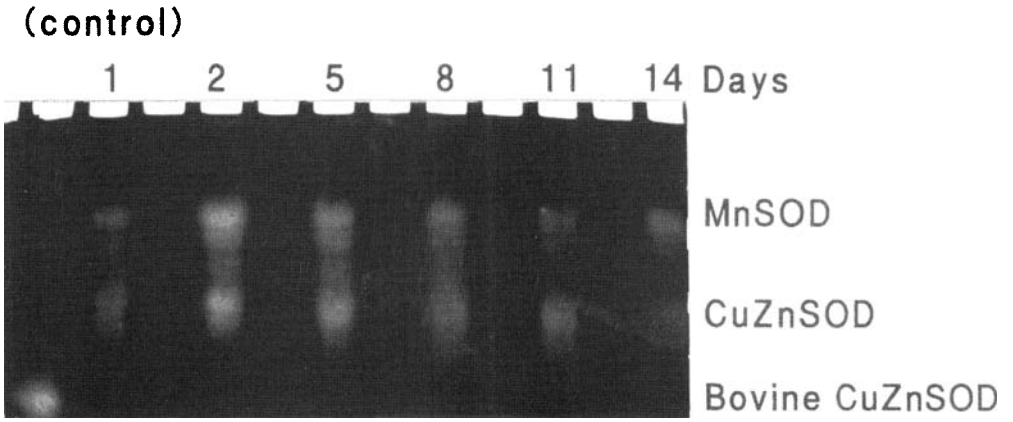

FIGURE 3 SOD activity assayed in the liver of guinea pig infected with *L. interrogans* on non-denaturing polyacrylamide gel. Numbers indicate the assayed days after infection with *L. interrogans* including control (number 1). Each lane received 40 μg of total protein. Bovine CuZnSOD was used as a standard (Sigma).

damage, protein damage) and the protective role of other antioxidants are needed to confirm the role of oxyradicals in this infection. These types of studies may provide insight into the mechanism involved in leptospira toxicity.

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