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# Effects of Medium Molecular Weight Heparinyl Phenylalnine on Superoxide Dismutase Activity in Mice

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**Abstract.** *We investigated the radical scavenging ability of heparin (HE), medium molecular weight heparinyl phenylalanine (MHF) and medium molecular weight heparinyl leucine (MHL) in the blood of mice. The extracellular superoxide dismutase (EC-SOD) activity was measured according to the method by Oyanagui and Sato. As a result, HE significantly increased the EC-SOD activity with a significant prolongation of activated partial thromboplastin time (APTT), while MHF and MHL significantly increased the EC-SOD activity without a prolongation of APTT. Dose-response curve at 20 min after the injection of each compound indicated a bell-shape. Changes in the plasma EC-SOD activity of mice after the administration of HE, MHF and MHL (10 mg/kg/10 ml) were investigated time-dependently. The plasma EC-SOD activity peaked at 5 min after the administration of all compounds. These results indicated that MHF and MHL show a radical scavenging ability by increasing the EC-SOD activity and MHF may be a candidate for clinical use.*

In our previous studies, we reported that medium molecular weight heparinyl phenylalanine (MHF) and medium molecular weight heparinyl leucine (MHL) have preventative effects against cultured human umbilical vein endothelial cells (HUV-ECs) damaged by oxygen-free radicals and ischemic paw edema in mice without prolonged activated partial thromboplastin time (APTT). MHF and MHL are superior to heparin (HE) as safe radical scavengers (1, 2). However, the mechanism of MHF and MHL as radical scavengers is unknown. In this study, we examined the change in the plasma extracellular superoxide dismutase (EC-SOD) activity after intravenous administration of MHF and MHL to clarify how MHF and MHL function as radical scavengers.

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*Key Words:* Medium molecular weight heparinyl phenylalanine (MHF), extracellular superoxide dismutase (EC-SOD), mouse, radical scavenge, free radical.

## Materials and Methods

*Animals*. Specific pathogen-free male ICR mice (4 weeks old) were purchased from Japanese Charles River Ltd., (Yokohama, Japan) and used for the experiment after a one-week acclimation. The mice were maintained at 23±2˚C (room temperature) and 50±5% (relative humidity) under an artificial 12-hour light-dark cycle (7:00 on - 19:00 off). Food and water were given *ad libitum* during the experimental period. All procedures followed the office regulations for the Care and Use of laboratory animals approved by the animal experimentation committee of Fuso Pharmaceutical Industries Ltd., Japan (approval number: PDS9702).

*Materials*. MHF and MHL (mean molecular weight: 8,500-10,000), synthesized at Fuso Pharmaceutical Industries Ltd. Research and Development Center (Osaka, Japan), and bulk HE (Scientific Protein Laboratories, Waunakee, WI, USA) were used (3).

*Effects of HE, MHF and MHL on the plasma EC-SOD activity in mice*. HE, MHF or MHL was injected into the tail vein of mice at several doses (2.5, 5.0, 10.0, 20.0 and 40.0 mg/kg/10 ml). Twenty minutes later, 0.5 ml of blood was collected from the abdominal aorta using the heparinized injection cylinder. To avoid leakage of SOD from red blood cells by hemolysis, collected blood was kept in a test tube containing 2 ml of 0.25 M sucrose (including HE 10 U/ml) and cooled in ice until analysis (as a sample). Twenty μl of the sample, 2 ml of 0.25 M sucrose and 10 ml of saline were put in a test tube and mixed gently. The mixture was then centrifuged (1,000 × *g*, 10 min, 4˚C) and the upper layer removed. The EC-SOD activity in the upper layer was measured according to the method of Oyanagui and Sato (4). Data were indicated using nitrite units (NU/ml plasma) of SOD.

*Statistical analysis*. Data are represented as the means±standard error (S. E.) and significance was evaluated by analysis of variance (ANOVA) followed by the Spjotvoll and Stoline-test (corrected Tukey method) because the number of samples among the groups was different. The differences were assessed at a significance level of 0.05.

## Results

*Effects of HE, MHF and MHL on the plasma EC-SOD activity in mice*. When HE was administered into mice intravenously, the plasma EC-SOD activity increased significantly with doses of 2.5 mg/kg/10 ml or higher. The increase in the plasma EC-SOD activity peaked at a dose of 10.0 mg/kg/10 ml. The plasma EC-SOD activity of the 10.0 mg/kg/10 ml-administered group increased by approximately 4-times compared with the PBS-administered control group. Furthermore, HE, higher than 10.0 mg/kg/10 ml, did not show any significant increase in the plasma EC-SOD activity compared with the PBS-administered control group. In the groups administered MHF and MHL, the plasma EC-SOD activity increased from the doses of 2.5 to 5.0 mg/kg/10 ml. The peak values of the plasma EC-SOD activity of the groups administered MHF and MHL were approximately 5-times in the case of MHF and 4-times in the case of MHL compared with the control group. The dose of MHF and MHL-EC-SOD activity curve formed a bell-shape, similar to HE (Figure 1).

*Changes in the plasma EC-SOD activity after HE, MHF and MHL were administered to the mice until 120 min*. The plasma EC-SOD activities of mice peaked at 5 min after administration of HE, MHF and MHL (10 mg/kg/10 ml). At 5 min after the injection, the plasma EC-SOD activities of mice decreased until 120 min in the groups administered MHF and MHL. However, the plasma EC-SOD activities of mice administered HE did not change after 20 min (Figure 2).

## Discussion

Many researchers have already reported that plasma EC-SOD activity is increased by administration of HE in mammals, such as humans, porcine, bovine, canines, cats and mice (4-10). In this paper, we chose the mouse as an experimental animal to investigate the effects of HE, MHF and MHL on SOD activity *in vivo*. In general, Unit of SOD (Unit/ml) is obtained by the cytochrome *c* method of McCord and Fridovich (11). However, the cytochrome *c* method can only be applied to samples that have a strong radical scavenge ability. Furthermore, the procedure is very complex and a highly sensitive spectrophotometer is needed. In this experiment, we utilized Oyanagui and Sato's method (4). Their method is not complex, shows good color development and the necessary reagents are economical. In addition, the absorbance changes can be detected by a conventional spectrophotometer. Moreover, the sensitivity of this method is more than 3-times higher than the cytochrome *c* method (12).

First, the effects of several doses of HE, MHF or MHL on mouse plasma EC-SOD activity were investigated. The increase in the plasma EC-SOD activities of mice were confirmed after intravenous administration of HE, MHF or MHL. The dose-EC-SOD activity curves formed a bell-shape. In the case of HE, the plasma EC-SOD activity increased significantly at doses of 2.5 mg/kg/10 ml or higher with a prolonged APTT. On the other hand, the plasma EC-SOD activity increased from doses of 2.5 to 5.0 mg/kg/10 ml without any prolonged APTT in groups administered MHF and MHL (APTT was measured after HE, MHF and MHL were administered to mice as in reference (2)).

EC-SOD in the blood circulation forms a tetramer and has four HE-binding sites (4). This tetramer without any bound HE is the inactive form. However, EC-SOD is activated by binding of two HE to two binding sites and EC-SOD can connect with other EC-SODs using the empty binding sites. That is, a proper dose of HE activates EC-SOD, while an excess dose of HE cannot activate EC-SOD (4). We speculate that MHF and MHL may also function as HE as the dose-EC-SOD activity curves formed a bell-shape.

Barzu *et al*. studied the binding of HE and low molecular weight heparin fragments (CY 222; molecular weight range: 1,500-8,000) to human vascular endothelial cells. As a result, HE indicated a much higher affinity to the vascular endothelium than CY 222 and a fraction of bound HE was reported to be internalized by the vascular endothelium (13). According to their paper, HE may bind to the vascular endothelium with a high affinity and a fraction of bound HE is internalized by the vascular endothelium, while smaller amounts of MHF and MHL bind to the vascular endothelial cells. We surmise that this is the reason why MHF and MHL exhibited a higher ability than HE to activate EC-SOD after intravenous injection.

Second, we investigated the changes in the plasma EC-SOD activity after HE, MHF and MHL (10 mg/kg/10 ml) were administered to the mice until 120 min. The plasma EC-SOD activity of the mice peaked at 5 min after the administration and decreased gradually until 120 min. HE demonstrated a smaller degree of decrease in EC-SOD activity than MHF and MHL that did not change after 20 min. We speculate that MHF and MHL were broken-down more quickly than HE as the molecular weights of MHF and MHL are smaller than HE's. However, MHF possessed a higher ability than HE to increase EC-SOD activity until 60 min. Considering the reduced side-effects of MHF, this result suggests that MHF is superior to HE as a candidate for clinical use.

Recently, participation of free radicals is reported in several diseases, such as in the cardiovascular system (14), Alzheimer's disease (15), arteriosclerosis (16), myocardial infarction (17), cerebral infarction (18), Parkinson's disease (19), pancreatitis (20), glomerulonephritis (21), diabetes (22), cirrhosis (23), stomach ulcers (24), atopic dermatitis (25) and asthma (26, 27). Although the research on EC-SOD is advanced and several radical scavengers are under

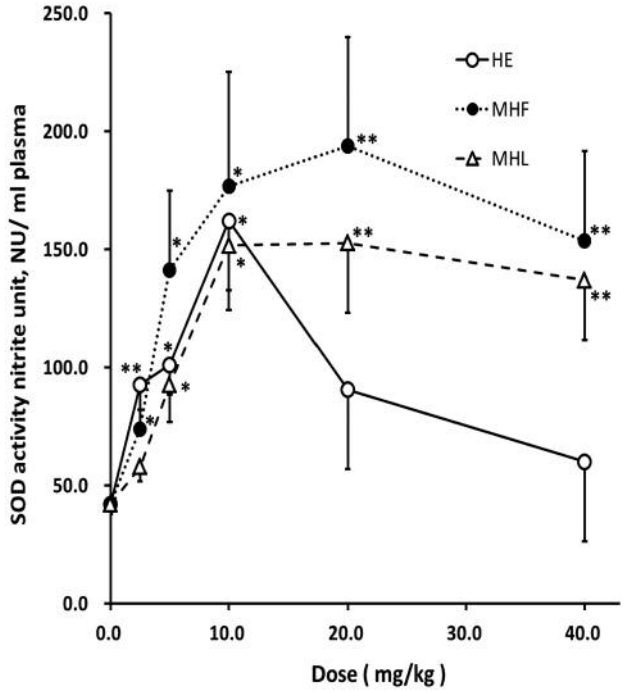


Figure 1. *Increase in the plasma extracellular superoxide dismutase (EC-SOD) activity of mice injected by several doses of heparin (HE), medium molecular weight heparinyl phenylalanine (MHF) and medium molecular weight heparinyl leucine (MHL) at 20 min after the injection. Each point represents the mean±S. E. of 8 or 11 animals. **p<0.01, *p<0.05 vs. PBS (by Spjotvoll and Stoline-test).*

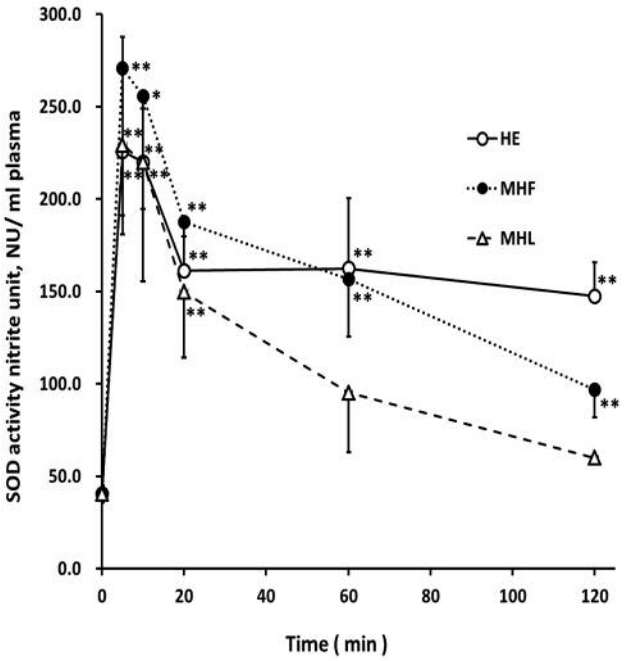


Figure 2. *Time course of plasma extracellular superoxide dismutase (EC-SOD) activities of mice after the injection of heparin (HE), medium molecular weight heparinyl phenylalanine (MHF) and medium molecular weight heparinyl leucine (MHL) (10 mg/kg/10 ml). Each point represents the mean±S. E. of 8 or 10 animals. **p<0.01, *p<0.05 vs. PBS (by Spjotvoll and Stoline-test).*

development, the number of ethical drugs is few. MHF may be a promising candidate for a radical scavenger to increase EC-SOD activity with a low risk of hemorrhage.

## Conflicts of Interest

The Authors declare no conflict of interest associated with this manuscript.

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