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# SUPEROXIDE DISMUTASES AND ANTI-OXIDANTS PROTECTED MICE FROM NO-REFLOW AND NECROTIC DAMAGE INDUCED BY ISCHEMIA

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A simple method in mice was established to screen anti-ischemic compounds. Thirteen times binding of rubber ring (1 × 1 mm, d = 42 mm) for 4.5 hrs, swelled the paws of 60% mice applied and 14 times binding swelled only of 5% mice. Critically reversible limit lay between these conditions. "All or none" rule dominated the paw swelling perhap due to different endogenous anti-oxidants' levels of individual mice. Determination of paw reversibility at 90 min of recirculation, was proved to be suitable. Swollen paws at this time returned normal and the paws with no-reflow dropped out by muscle necrosis after several days. Intravenous (i.v.) bovine Cu, Zn-SOD and bacterial Mn-SOD ($3 - 10 \times 10^4$ U/kg) or liposomal Cu, Zn-SOD ($0.3 - 3 \times 10^4$ U/kg) were protective (35-50%) by 14 times binding. Allopurinol (10-100 mg/kg) and D-mannitol (3-30 mg/kg) was effective (25-55%). Catalase (i.v., up to $10^5$ U/kg) showed little protection, but local injection of 100 U/kg resulted in 50% protection. Glutathione (30 mg/kg) was suppressive only by local injection suggesting the importance of administration route. Desferal, heparin and nitric oxide synthesis inhibitor showed some protection, but indomethacin, mepyramine, ascorbate, vitamin E and dexamethasone were without effect. Excess dosing of all anti-oxidants tested, dramatically decreased their effects demanding caution for therapeutic trials.

KEY WORDS: SOD: anti-oxidant, D-mannitol, allopurinol, no-reflow, ischemia, paw swelling, necrotic damage

Abbreviation SOD: superoxide dismutase, GSH : reduced glutathione, L-NMMA:L-$N^G$-monomethyl-arginine, GPX: glutathione peroxidase

## INTRODUCTION

Prevention of ischemia-reperfusion damage by reactive oxygen species ($O_2^-$, $H_2O_2$, $\cdot OH$, $OCl^-$) has been tried in various models in the isolated organs and in animals. We have previously observed SOD and some antioxidants reduced the paw swellings.[1] Commercial rubber ring was bound to make ischemia for 20 min and measured the paw swelling at 20 min after recirculation. In this model of short-time ischemia, paws always recovered its function as well as normal paw thickness after several days. Drugs were intravenously (i.v.) injected just before ischemia. SODs from various animals were suppressive in different potencies ($IC_{30}$ = 3-3,000 U/kg). Scavengers of $H_2O_2$ and hydroxyl radical ($\cdot OH$), indomethacin, mepyramine, glutathione, ascorbate were not effective. Xanthine oxidase (XOD) inhibitor, allopurinol showed $IC_{30}$ value of 0.3 mg/kg. Dexamethasone suppressed only when injected more than 3 hrs in advance ($IC_{30}$ = 30 μg/kg). However, these

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results seemed not to represent the effect of drug in many clinical situation. For example, coronary occlusion could rarely re-opened within 20 min. We prolonged the ischemic time and increased the binding times of rubber ring in mice. These ischemic load arrived to cause no-reflow phenomenon followed by necrosis. We found a way to predict the consequences of ischmia-exposed paws at 90 min of recirculation which required less time and gave less suffering to the animals. Drugs (i.v. or local) were tested in this way finding some differences with the result by short-time ischemia.



## MATERIALS AND METHODS

### *Animals*

Male ddy mice (5 weeks old) were obtained from SLC Co. (Shizuoka, Japan). Animals were kept in air-conditioned room for at least 1 day. They were weighed 1–3 hrs before the experiment for selecting 27–33 mice.

### *Assay*

Commercial rubber ring (1 × 1 mm, d = 42 mm) was used for making ischemia. A mouse was placed in a plastic cylinder device being picked up by the right leg from the slit. Mouse was bound by rubber at just above the articulation with equal strength as possible and kept in cage. The rubber was scissored off after the indicated time using the same device. Paw thickness was measured with Citizen thickness gauge KG-1 (Citizen Watch Co., Tokyo) at different time of natural recirculation. Paws which did not swell at 90 min of recirculation never returned to the normal thickness to function, and therefore we counted the number of mice with swollen paws in rose as non-damaged or protected individuals (Figure. 1). Each experiment was performed with 40–100 mice always including a control group. Enzymes and agents were dissolved in saline and injected just before ischemia into the tail vein (0.5 ml/30 g body weight) for intravenous (i.v.) dosing, In the case of local injection in paw, a micro-injector for HPLC was applied. Allopurinol was first dissolved in a little amount of 1 M NaOH and diluted with saline followed by adjustment to pH 7 by 1 M and 0.1 M HCl. Indomethacin and vitamin E was at first in pure N,N′-dimethylformamide (DMF) and diluted with saline to make 5% of DMF solution which had no significant effect in paw swelling. Salts and crystal waters were included for calculation of drug doses.

### *Chemicals*

Bovine erythrocyte Cu, Zn-SOD (3,000 U/mg protein), $N^G$-monomethyl-L-arginine · acetate (L-NMMA) and adenosine triphosphate ·2 Na (ATP) were obtained from Sigma Chem, Co. (St. Louis, USA.). Liposomal bovine blood Cu, Zn-SOD (3,600 U/mg protein SOD, 1 mg in 6 mg solid) were kindly offered from Dr. Michelson (Paris) through Dr. Niwa (Tosashimizu, Japan). Bacterial (*Bacillus stearothermophilus*) Mn-SOD (8,200 U/mg protein) were purchased from Unitika Co. (Kyoto). Bovine liver catalase (1,500 U/mg solid) was from Tokyo Kasei Co. (Tokyo). Dexamethasone (Decadron, 3.3 mg/ml sodium phosphate) and desferroxamine · mesylate (Desferal), was from Japan-Merck Co. and Ciba-Geigy Co.

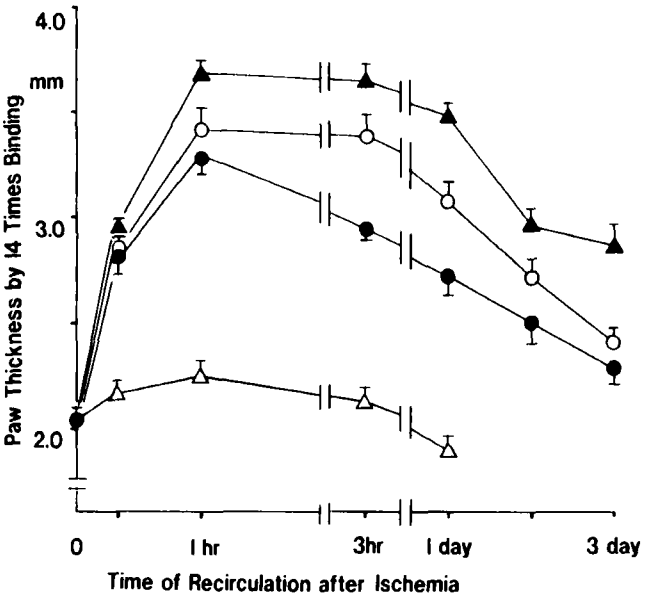


FIGURE 1 Time-course of paw swelling after different time of ischemia by 14 times binding of rubber ring. Ischemia was for 20 min (●), 1 hr (▲), 2 hr (○) and 5 hr (△). Vertical lines represent standard error (S.E.) of mean of 6 mice.

(Takarazuka, Japan), respectively. Indomethacin was synthesized in this laboratory. Reduced glutathine (GSH), D-mannitol, allopurinol and the other drugs of possibly pure grade were purchased from Nakarai Chem, Co. (Kyoto).

## RESULTS

### *Time-Course of Paw Swelling*

Short time of ischemia (20 min) by 14 times rubber ring binding, caused swelling at 1 hr of recirculation followed by its rather rapid reduction (Figure 1). Prolonged ischemia (5 hrs) failed to swell the paw. Since the ischemia of 1 or 2 hrs resulted in the swelling to make plateau between 1 and 3 hr of recirculation, we decided to test the effect of anti-oxidants at 90 min of recirculation. There were 3 types of paw appearances at 90 min: rose swollen, dark swollen (rare case) and dark or pale non-swollen. Only the first type could return to normal thickness with function after several days. We counted only this first type of paw as positive protected one. The other 2 types of paws became blackish, thin and dry dropping off finally.

### *Effect of Ischemic Strength*

Binding 12 times swelled the paw of 4 out of 10 mice even after 6 hrs' ischemia (Figure 2). Non of another 13 mice showed paw swelling by binding 16 times for 3 hrs. Any ischemic strength tested caused non-reversible damage as far as ischemia was less than 2 hrs. Five hrs of ischemia by more than 13 times binding rendered

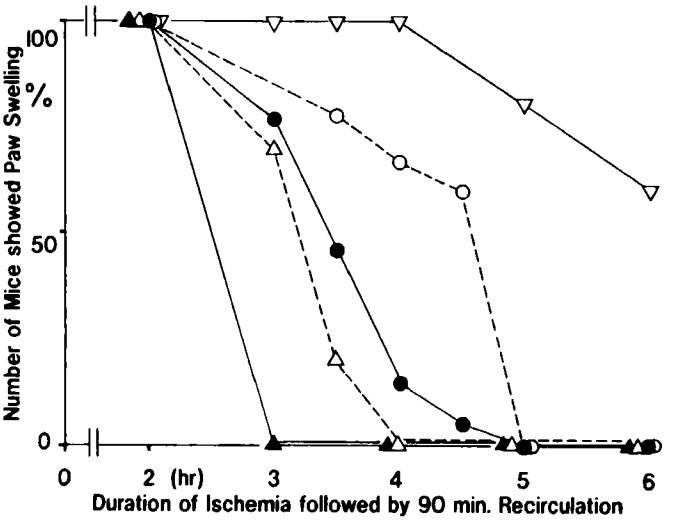


FIGURE 2 Percentage of mice with paw swelling at 90 min of recirculation after different strength of ischemia. Binding by rubber ring was 12 times (▽; n = 5, 5, 5, 5, 10, 10 from the left), 13 times (○; n = 5, 9, 15, 20, 10, 5), 14 times (●; n = 10, 13, 14, 20, 20, 10, 5), 15 times (△; n = 5, 10, 20, 15, 5, 5) and 16 times (▲; n = 5, 13, 10, 10, 5).

all mice impossible to swell their paws. Additional once binding over 13 times bound mice, was critical for reversibility of their paws; 12 out of 20 mice (60%) which were received 13 times binding for 4.5 hrs reversibly swelled their paws. However, only 1 out of 20 mice (5%) of 14 times binding succeeded to swell its paw. As complete impairment of paw swelling seemed difficult to be ameliorated by any treatment, 14 times binding for 4.5 hrs' ischemia was adopted for testing anti-oxidative enzymes and other agents.

### *Protection by Intravenous Anti-Oxidant Enzymes*

Intravenous Cu, Zn-SOD ($3 \times 10^4$ U/kg) increased the number of swollen paw up to 49% by 14 times binding for 4.5 hrs (Figure 3). Liposomal Cu, Zn-SOD and bacterial Mn-SOD also increased the number of mice with swollen paws. Protection by catalase was minor but evident. Mixture of Cu, Zn-SOD and catalase did not show the protection more than Cu, Zn-SOD alone. Nine out of 15 mice by $10^4$ U/kg of both enzymes swelled their paws and none of 15 mice showed their paw swelling by $10^5$ U/kg of both enzymes. Control groups of at least 5 mice were used in every experiments confirming the previous result; for example, only 3 out of 75 mice (4%) in total showed swellings by 14 times binding for 4.5 hrs. However, these data were fragmentary and the control curve of Figure 2 was inserted to Figure 3 and 4.

### *Protection by Intravenous Anti-Oxidants*

Hydroxyl radical scavenger, D-mannitol (10 mg/kg) protected the paw (50%) (Figure 4). Effect of XOD inhibitor, allopurinal (30 mg/kg) was a little more potent and nitric oxide synthesis inhibitor, L-NMMA (100 mg/kg) was less protective.

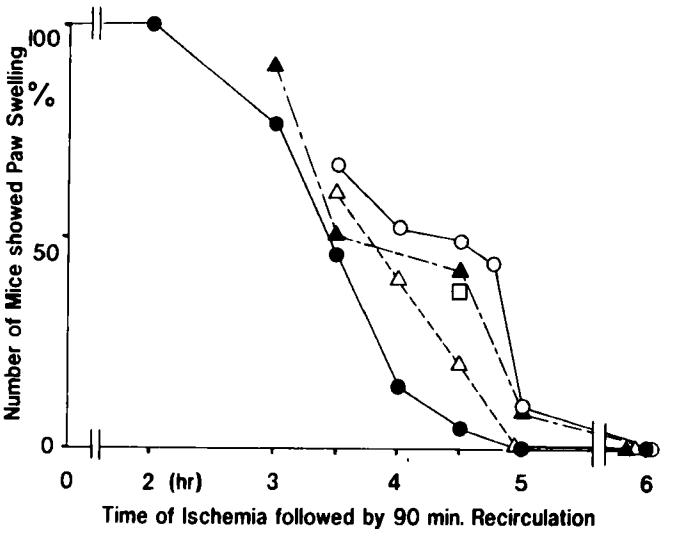


FIGURE 3 Increased percentage of mice with paw swelling by anti-oxidant enzymes (i.v.). Ischemia was by 14 times binding for 4.5 hrs. Control (●; n = 5, 13, 14, 20, 20, 10, 5 from the left), Cu, Zn-SOD $3 \times 10^4$ U/kg (○; n = 12, 12, 39, 15, 10, 5), liposomal Cu, Zn-SOD $3 \times 10^3$ U/kg (▲; n = 5, 10, 18, 13, 10), catalase $10^5$ U/kg (△; n = 10, 10, 10, 10, 5) and bacterial Mn-SOD $3 \times 10^4$ U/kg (□; n = 19).

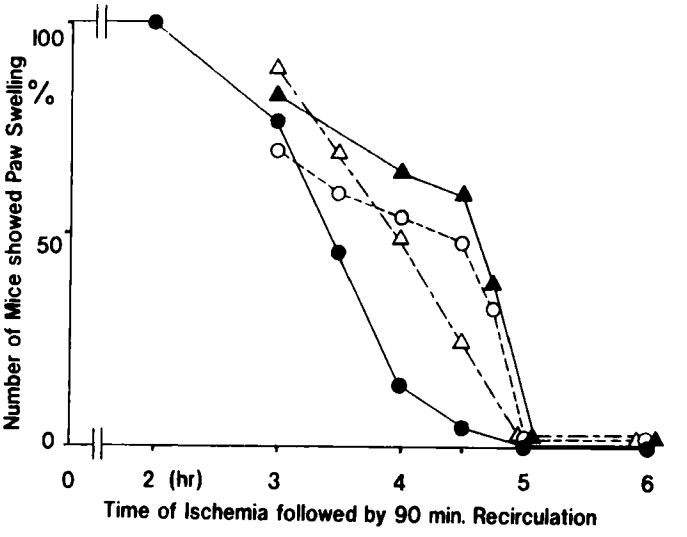


FIGURE 4 Increased percentage of mice with paw swelling by anti-oxidant agents (i.v.). Ischemia was by 14 times binding for 4.5 hrs. Control (●; n = as in Figure 3), allopurinol 30 mg/kg (▲; n = 10, 11, 10, 13, 10, 10) , D-mannitol 10 mg/kg (○; n = 10, 11, 19, 16, 10, 10, 5 and L-NMMA 100 mg/kg (△; n = 10, 10, 10, 20, 10, 5).

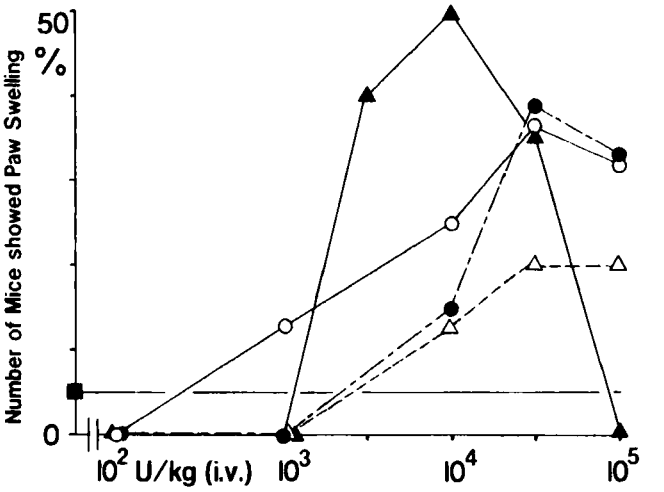


Figure 5 Dose-response curve of anti-oxidant enzymes (i.v.). Ischemia was by 14 times binding for 4.5 hrs. Control (■; n = 20), Cu, Zn-SOD (●; n = 5, 10, 20, 18, 15, from the left), liposomal Cu, Zn-SOD (▲; n = 5, 10, 10, 10, 10, 10), catalase (△; n = 8, 8, 10, 10) and bacterial Mn-SOD (○; n = 4, 8, 10, 8, 6).

### *Dose Dependency of Intravenous Enzymes*

Biphasic protection seemed to be common for all intravenously dosed anti-oxidants. Liposomal Cu, Zn-SOD was most effective by $10^4$ U/kg, but ten times more or less dose were without effect (Figure 5). Optimum dose of Cu, Zn-SOD and bacterial Mn-SOD were $3 \times 10^4$ U/kg resulting in about 40% protection. Catalase could not exceed 20% protection by any dose. Allopurinol (30 mg/kg) and D-mannitol (10 mg/kg) increased number of mice with reversible swelling but less effectively by higher doses (Figure 6). Effect of GSH and L-NMMA were not remarkable.

### *Non-Protective Agents*

Various agents were tested being found little or no protection. Ascorbate (i.v.) had almost no effect (3/12 of swollen paw by 100 mg/kg and 0/9 mice by 10 mg/kg). Vitamin E (i.v. and local) showed no effect by 100 and 10 mg/kg (n = 8). Arachidonic acid metabolites had no role in our model as indomethacin and phenidone (30 and 3 mg/kg, i.v.) protected no mice (n = 8–10). Mepyramine (100 and 10 mg/kg) was also without effect (n = 10–12). Dexamethasone (3 mg/kg, i.v.) failed to protect the paws either injected just before or 3 hrs before ischemic onset (n = 10). Heparin (2,000 and 1,000 U/kg, i.v.) swelled 2 paws out of 12 (17%) and none of 16 mice by 100 mg/kg. Another anti-coagulant, sodium citrate (i.v.) was without effect (0/8 by 100 and 10 mg/kg). Iron chelator, Desferal (i.v.) showed a little protection (2/13, 3/13 and 3/15 mice by 100, 30 and 10 mg/kg, respectively). ATP (30 and 10 mg/kg, i.v. and local) was also ineffective (n = 8–10).

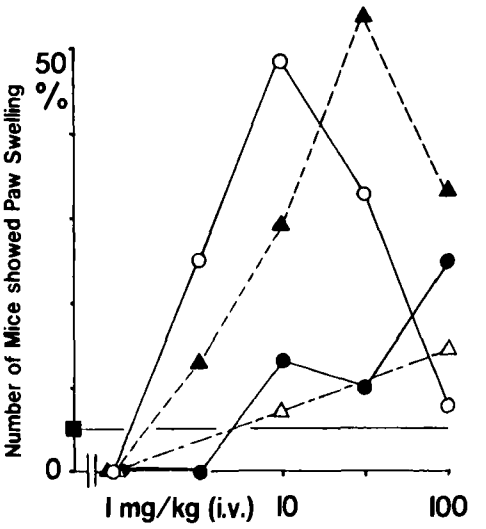


FIGURE 6 Dose-response curve of anti-oxidant agents (i.v.). Ischemia was by 14 times binding for 4.5 hrs. Control (■; n = 20), D-mannitol (○; 8, 8, 12, 12, 12 from the left), allopurinol (▲; n = 8, 8, 16, 16, 8) GSH (●; n = 4, 8, 8, 10, 8, and L-NMMA (△; n = 8, 8, 8).

### *Dose Dependency of Anti-Oxidants by Local Injection*

Catalase (100 U/kg), which had no protective effect by intravenous injection, swelled paws of 50% mice by local injection (Figure 7). Cu, Zn-SOD was also more potent by local injection (50% protection by $10^4$ U/kg). Dose-response curve of allopurinol by local injection resembled very much to that by intravenous injection. GSH protected significantly the paws at the dose more than 10 mg/kg by local injection in spite of its very weak effect by intravenous dosing. Many enzymes and agents must have inactivated or had difficulties to arrive to the target tissue by intravenous injection.

## DISCUSSION

Screening of anti-ischemic compounds *in vitro* has limited value and that *in vivo* with mice offers more valuable informations requiring little sample, simple manipulation and low cost. Tissue responsible to our ischemic paw model is mainly muscles. Bones and skins might participate little. Though we did not measure the blood flow or pressure and local oxygen tension, the edema formation after ischemia must have accompanied lowering of blood circulation. Severe ischemia caused no-reflow phenomenon followed by necrosis. Time course of intramuscular pressure of rat extensor hallucis proprius after 60 min of ischemia,[2] resembled to that of our results in Figure 1. Renal blood flow of rat at 1 hr of recirculation after 30 min and 60 min of ischemia was 65% and 25% of control.[3] Lowered oxygen tension predicted the ulceration or gangrene of lower limbs in patients with circulation disorders.[4] Bones might not be attacked at our observation time (90 min of

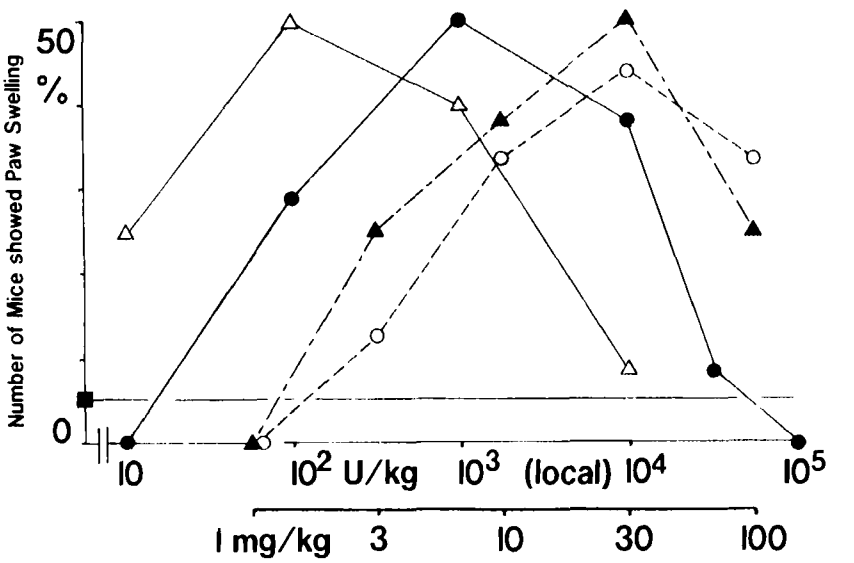


FIGURE 7 Dose-response curve of locally (in paw) injected anti-oxidant enzymes and agents. Ischemia was by 14 times binding for 4.5 hrs. Control (■; n = 20), Cu, Zn-SOD (▲; n = 4, 7, 16, 12, 4 from the left), catalase (△; n = 8, 12, 12, 11), allopurinol (●; n = 4, 9, 13, 9, 9, 8), and GSH (○; n = 8, 8, 9, 8, 8).

recirculation) as hemodynamic disorders appeared enough before radiographical abnormality of bones.[5]

Any dose of SODs on ischemia by 14 times binding could not overcome the reversibility of 13 times binding's control. This degree of protection by SOD corresponded to that observed in rabbit ear necrosis after freezing.[6] Bovine Cu, Zn-SOD (3,000 U/mg, 6 mg/kg bolus i.v. followed by infusion of 4 mg/kg/min) caused only 30% necrotic area at 7th day after 60 second freezing of ear when control resulted in 75% necrosis. However, this dose of SOD was not effective when ear was dipped in dry ice-ethanol ( − 21 °C) for 120 second (100% necrosis in both control and SOD groups). Cu, Zn-SOD (20,000 U/kg, i.v.) is also reported to show diminished necrotic area of rat skin flap (39% versus 59%).[7] In contrast, Ricci *et al.*[8] observed that albumin conjugated Cu, Zn-SOD (6,000 U/kg, i.v., 30 min in advance) did not preserve normal neuromuscular function despite a significant reduction in muscle damage after limb ischemia in dogs.

Excess dosing of SOD is now well recognized to be less protective in many animal models. This was true in our previous experiment of paw edema by short time ischemia for Cu, Zn-SODs from bovine, human, rat, mouse and guinea pig erythrocytes.[1] We found here again the bell-shape protection curve by SODs (Figure 5). The most drastic was by liposomal Cu, Zn-SOD; 10,000 U/kg (i.v.) protected 50% of mice from blocking of recirculation of paws, but 10 times more amount showed completely no effect. Combination of catalase with Cu, Zn-SOD (i.v.) had no additional effect (data not shown) suggesting no possibility that $H_2O_2$ inactivated injected Cu, Zn-SOD. There is not yet an answer for this decreased biological effect of SOD, but Omar *et al.*[9] proposed a possible mechanism to explain their result that 50 mg/kg Cu, Zn-SOD protected ischemia-induced infarct size of isolated

rabbit heart less than half by those of 2.3, 7 and 20 mg/kg. They mentioned that overscavenging of $O_2^-$ ($HO_2\cdot$ in acidic condition) by increased amount of SOD would eliminate an important termination step of lipid peroxidation ($LOO\cdot + HO_2\cdot \rightarrow LOOH + O_2$) and thus exacerbate the damage.

Intravenously administered catalase minimally protected the paw, but local injection was very effective by 100 U/kg (Figure 7). There is no clear interpretation for this difference except possible inactivation of this enzyme in blood stream. Large variation in human serum catalase activity is reported as a cause of different susceptibility among individuals to oxidant-mediated vascular diseases.[10] We loaded the same ischemic stress to mice finding different result between individual mice (swelling and non-swelling paws) which might be due to this kind of difference of anti-oxidant enzyme (s). Laughlin *et al.*[11] found increased oxidative capacity, increased glutathione peroxidase (GPX) activity and decreased catalase activity in forelimb muscles of exercised trained rats (2 hr/day treadmill, 5 days/week for 12 weeks). Exposure of this trained rats to 1 hr ischemia-1 hr reperfusion resulted in decreased GPX and increased catalase activity than sedentary rats. Changes of GPX and catalase activities seemed to depend on the type of stimulus and conditions.

Hydrogen peroxide is injurious in many cases as pro-oxidant to form very reactive hydroxyl radical ($\cdot OH$) under heavy metal ions. In fact, $\cdot OH$ scavenger, D-mannitol (10 mg/kg, i.v.) protected our ischemic paws of mice showing the decreased effect by higher doses (30 and 100 mg/kg) (Figure 6). Another $\cdot OH$ scavenger, dimethylsulfoxide (DMSO, 30 min reperfusion by 5% solution) suppressed the vascular permeability of canine gracilis muscle by 4 hr ischemia-4 hr reperfusion.[12] Lacks of protection by histamine receptor antagonists in this report were in accordance with our result that mepyramine had no effect. Local injection of GSH protected more efficiently our ischemic paws than intravenous injection. GSH can remove $H_2O_2$ and another thiol compound, dihydrolipoic acid (8 $\mu$M) restored the flexibility of the isolated rat hindlimbs after 4 hr ischemia −30 min reperfusion.[13] An anti-oxidant, coenzyme $Q_{10}$ (10 mg/kg, i.v.) protected the damage of isolated canine gracilis muscle from ischemic damage.[14] Myoglobin and $H_2O_2$ promoted the lipid peroxidation of unsaturated fatty acid and caused damage which could be protected by ascorbate.[15] However, we failed, to protect our ischemic paws by both i.v. and local injection of ascorbate. There might not be enough metmyoglobin in our models or another unknown mechanism impaired the effect of exogenous ascorbate.

Vitamin E was not protective with our fine homogenous suspension. ATP (i.v. and local injection) was also ineffective in our model though Lindsay *et al.*[16] claimed that the degree of the ischemic ATP degeneration might be the most important determinant of the ultimate extent of skeltal muscle necrosis. Inability to supply exogenous ATP to the adequate site of muscle, might be the cause of our negative result. Some effect of Desferal (iron chelator) could be supported by the work in rat skin flap model after 7 hrs' occlusion.[17] Anti-coagulant heparin was a little protective, but sodium citrate had no effect. Negatively charged heparin might have liberated extracellular (EC)-SOD to protect ischemic paws. Paw swellings after short time ischemia of mice was reduced by intravenously administered heparin.[18]

Recent discoveries that nitric oxide ($NO\cdot$) damaged cells made us to try inhibitor of NO synthesis, L-N $^G$-monomethyl-arginine (L-NMMA) finding some protection. Endothelium XOD surely played important role to damage paw muscles as allopurinol (i.v. and local) suppressed significantly the damage of ischemic paws. Gastrocnemius muscle of rat after 3 hr ischemia-4 hr reperfusion[19] and gracilis

muscle of dog after 4 hr ischemia-30 min reperfusion[20] were concluded to be caused by neutrophil accumulations. Piroxicam, a non-steroidal antiinflammatory drug inhibited $O_2^-$ production by phagocytes and its oral dosing of 20 mg/day for 15 days recovered more rapidly the normal level of blood oxidant activity increased by treadmill exercise in patients with claudication.[21] In contrast, no participation of neutrophils was also concluded for no-reflow phenomenon of rat hind limbs after 4 hr ischemia-5 hr reperfusion.[22] We can not allude to the role of neutrophils in our model as no special trial was performed.

In conclusion, anti-oxidants protected the paw damage of mice induced by prolonged ischemia. There was a critical point in time and in degree of ischemia for reversibility of the tissues at risk. Exogenous anti-oxidants could ameliorate the paws only under the narrow range near this critical point. Route of administration (i.v. or local) was important to attain a good protection. The best route might depend on nature of each enzymes and agents. All anti-oxidants tested protected the paws in biphasic manner. Excess dosing decreased or lost their effects and this fact must be well considered for the,therapy by anti-oxidants. Protection by anti-oxidant followed to the rule of "all or none" as well as tolerance against ischemia of normal mice. Whether this difference among individuals comes from their different amount of endogenous anti-oxidants or from different rapid inducible capacity of unknown compound (s) like heat-shock protein (s), is a target for further research.

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