Int J Leg Med (1992) 105:5–9

International Journal of
Legal Medicine

Original articles

Suppression of ischemia-reperfusion injury by liposomal superoxide dismutase in rats subjected to tourniquet shock

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Received December 4, 1991 / Received in revised form February 18, 1992

Summary. To investigate the role of oxygen-derived free radicals in the pathogenesis of tourniquet shock, the authors present an experimental animal model. Two groups of rats were fastened with rubber tubes on both thighs (1.5 kg/cm^2) for 6 h under pentobarbital anaesthesia. One group was administered liposomal superoxide dismutase (L-SOD 30,000 U/kg body weight), and the other liposome as a control 3 h prior to tourniquet removal. No rats in the contorl group ($n = 20$) survived more than 24 h after reperfusion, whereas 55% of animals treated with L-SOD ($n = 20$) survived for 24 h or more, and two recovered completely ($P < 0.005$). Blood samples were obtained from the abdominal aorta after laparotomy of anaesthetized rats of both groups at different time intervals. Changes in the hematocrit value and blood urea nitrogen during the early periods after reperfusion were attenuated by prior administration of L-SOD, and the total plasma SOD activity of the control animals decreased promptly and continuously throughout the experimental period. This experimental model was very useful to study the pathogenesis of tourniquet shock with respect to reproducibility, induction of the shock stages and mortality. It is thought that oxygen-free radicals are involved in the induction of tourniquet shock, and L-SOD was, to a certain extent, effective against reperfusion injury in the early stages of shock.

Key words: Tourniquet shock – Ischemia-reperfusion injury – Liposomal superoxide dismutase – Survival interval

Zusammenfassung. Um die Rolle O_2-bedingter freier Radikale in der Pathogenese des Tourniquet-Schocks zu untersuchen, stellen die Autoren ein experimentelles Tier-Modell vor. Unter Pentobarbital-Anästhesie wurden zwei Gruppen von Ratten an den Schenkeln der Hinterläufe mit Gummischläuchen ligiert (1,5 kg/cm^2) – der Zeitraum betrug 6 Stunden. Eine Gruppe erhielt liposomale Superoxid-Dismutase (L-SOD 30,000 U/kg Körpergewicht), die andere zur Kontrolle eine Liposom-Lösung; beide Lösungen wurden drei Studen vor Lösung des Tourniquet-Schocks in die Schwanzvene injiziert. In der Kontrollgruppe ($n = 20$) überlebte keine der Ratten mehr als 24 Stunden nach Reperfusion, während 55% der mit L-SOD behandelten Tiere ($n = 20$) mehr als 24 Stunden überlebten, zwei Tiere erholten sich sogar vollständig ($P < 0{,}005$). Blutproben wurden aus der Bauch-Aorta der anästhesierten Tiere nach Laparotomie und zu verschiedenen Zeitpunkten entnommen. Veränderungen im Hämatokrit-Wert und im Harnstoff N des Blutes während der frühen Phase nach Reperfusion wurden durch die vorherige Gabe von L-SOD verringert. Die gesamte SOD-Aktivität der Kontrolltiere nahm rasch und kontinuierlich während des Experiments ab. Das experimentelle Modell war sehr nützlich, um die Pathogenese des Tourniquet-Schocks mit besonderer Berücksichtigung der Reproduzierbarkeit, der Induktion der Schockzustände und der Mortalität zu untersuchen. Es ist daran zu denken, daß sauerstofffreie Radikale bei der Induktion des Tourniquet-Schocks beteiligt sind und daß bis zu einem gewissen Ausmaß L-SOD gegen den Reperfusionsschaden effektiv ist, letzteres in der frühen Phase des Schocks.

Schlüsselwörter: Tourniquet-Schock – Reperfusionsschaden – Liposomale Superoxid-Dismutase – Überlebenszeit

Introduction

Although destruction of vital organs and structures does not occur, serious bodily injury may cause delayed death from complications of the original injury such as hemorrhage, infection or traumatic shock. In such cases the relationship between the injury and death may be a point

A part of this work was presented at the 75th Congress of the Medico-Legal Society of Japan, Kyoto, April 1991.

The authors carried out the research described in this report in accordance with The Guide for Animal Experimentation, Tohoku University School of Medicine.

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in dispute both for civil compensation and criminal responsibility. Therefore, the investigation of post-traumatic complication is one of the most important issues in forensic pathology.

Tourniquet shock has often been employed as an experimental model for traumatic shock [1–6]. Several factors, including the release of biologically active substances and toxic compounds from the crushed or ischemic tissue have been suggested as possible causes for the induction of the shock.

However, recent work indicates that oxygen-free radicals could be implicated in the pathogenesis of various shock states [7–11]. The generation of these highly reactive molecules is increased under critical conditions related to shock such as tissue hypoxia and complement-induced granulocyte aggregation.

It seems that over-generation of superoxide radicals occurs during tourniquet shock, because the experimental animals were subjected to transient tissue ischemia of the extremities followed by re-oxygenation. Therefore, the development of tourniquet shock would be directly associated with the ischemia-reperfusion injury. In this experiment, we have studied the effects of L-SOD on the survival rate and on some biochemical changes in blood in the early stages of shock.

Material and methods

Animals. Female albino rats (Wister-Imamichi strain) weighing betwen 250 and 280 g were used. The rats were kept in clean cages and allowed food and water *ad libitum*.

Enzyme. Liposomal superoxide dismutase (L-SOD) and vacant liposome as a control reagent were gifts from Tosoh Co. (Tokyo Japan). The enzyme was encapsulated with cationic liposome and prepared from bovine erythrocytes [12].

Assay of SOD activity. Serum SOD activity was measured by the nitrite method described by Elstner and Heupel [13] and modified by Ōyanagui [14]. Briefly, hydroxylamine, xanthine oxidase, hypoxanthine and EDTA were incubated at pH 8.2, and 37°C for 30 min. A diazo reagent was added and the absorption measured at 550 nm.

Experimental protocols. The tourniquests consisted of rubber tubes (internal diameter 4 mm, external diameter 5.8 mm). After being anaesthetized with pentobarbital, both thighs of the rats were fastened by the rubber tubes and the pressure, which was monitored by a miniature pressure sensor (diameter 6 mm, Kyowa Electronic Instruments Co. PS-2KAMI92, Tokyo) and an amplifier (Kyowa Electronic Instruments Co. WGI-300A-1, Tokyo), was adjusted to 1.5 kg/cm^2. The rubber tubes were knotted and the sensor was removed. The tourniquet was left in place for 6 h and the animals remained under pentobarbital anaesthesia throughout. Afterwards, the rubber tubes were removed, and the rats were returned to their cages. Within a few minutes, the reperfused hindlimbs, which had been pale blue, turned pink, and the animals were then allowed free access to food and water. The rats received a single dose of L-SOD (30,000 U/kg body weight) or the vacant liposome injections in the tail vein 3 h prior to the release of the tourniquet. Hematocrits, plasma SOD, glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN) and total protein were monitored in a separate group of rats ($n = 80$) which were also divided in 2 groups. The measurements were carried out at different times (just prior to the release, 1, 2, 3 and 6 h after the release, $n = 8$ each) from samples of heparinized blood obtained from the abdominal aorta after laparotomy of anaesthetized rats.

Statistical analyses. Statistical evaluation of the survival interval was performed with the log lank test proposed by Peto et al. [15, 16]. Blood biochemical data were analyzed using the Kruskal-Wallis rank sum test [17]. Multiple comparisons were performed by Bonferroni's method [18].

Results

Survival interval and survival rate

In our model, 50% of the control animals died within 12 h after release, and none survived more than 24 h (Fig. 1). When administrated with L-SOD prior to release, 100% of rats survived more than 12 h, 55% more than 24 h and two recovered ($P < 0.005$). The control animals showed constriction of peripheral vein and hypothermia 1–2 h after the tourniquet removal. All animals without any administration ($n = 8$) died within 24 h (data not shown).

Hematocrits and plasma total proteins

The hematocrits of the control animals increased promptly and reached a maximum 2 h after the release and then leveled off (Fig. 2 top). The maximum value was approximately 30% higher than that of the normal control without tourniquet application. In the animals pretreated with L-SOD, the hematocrits increased gradually up to 6 h after release and reached the level of the control animals. Significant statistical differences were observed between the values of the initial 2 h for both groups ($P < 0.01$ at 1 h and $P < 0.05$ at 2 h after the release). Contrariwise, the total plasma proteins of both groups decreased by about 10% one hour after tourniquet removal and remained at that level (Fig. 2 bottom). No significant differences were shown between each group throughout 6 h of observation after the release. Both values of hematocrit and total protein did not elevate during the tourniquet application.

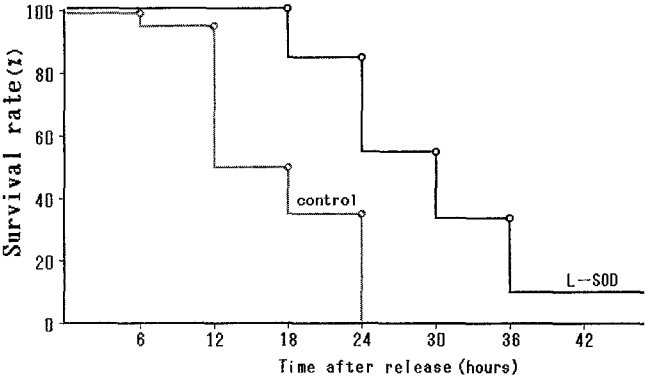

Fig. 1. Survival rates of rats subjected to tourniquet shock. Dotted lines, non-treated controls ($n = 20$); solid lines, liposomal superoxide dismutase (L-SOD) pretreated animals ($n = 20$). Statistical comparisons between the groups: $P < 0.005$

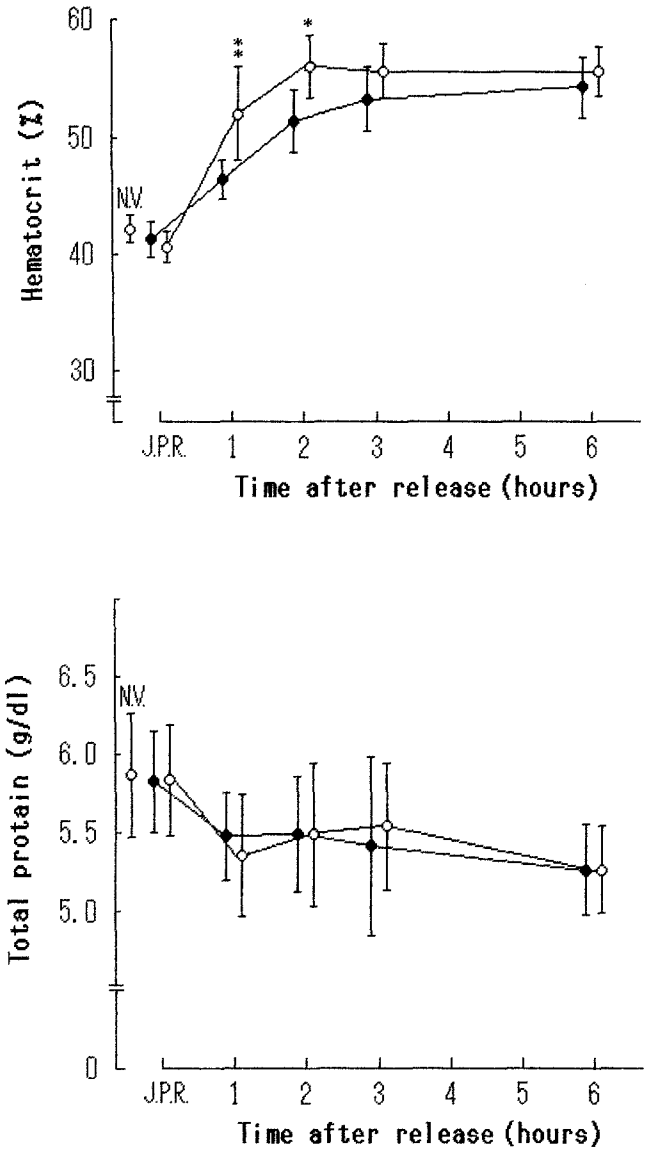

Fig. 2. Changes in hematocrit *(top)* and plasma total protein *(bottom)* level after tourniquet removal. *Circles,* non-treated controls ($n = 8$ each time); *dots,* liposomal superoxide dismutase (L-SOD) pre-treated animals ($n = 8$ each time). Values represent mean ± SE. J.P.R.: just prior to the release. N.V.: normal value of rats not subjected to tourniquet application. Significance between the groups on hematocrit level is shown. ‡: $P < 0.01$, *: $P < 0.05$

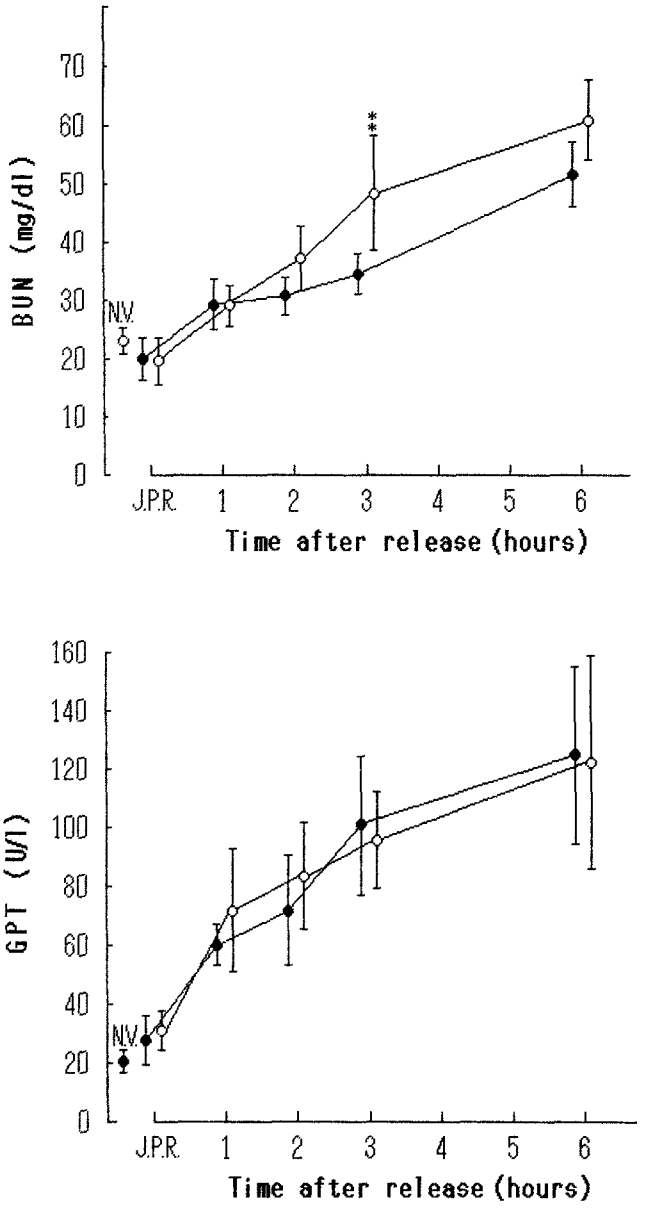

Fig. 3. Changes in plasma BUN *(top)* and GPT *(bottom)* level after tourniquet removal. Abbreviations as in Fig. 2. ‡: $P < 0.01$

Plasma BUN and GPT values

The BUN value of both groups of animals increased immediately after the release to a certain extent (Fig. 3). In the L-SOD pretreated animals the increase of the value attenuated during 1–3 h after tourniquet removal, but remained stable in the control animals ($P = 0.001$ at 3 h after the release). The BUN value of the L-SOD pretreated animals increased again and approached that of the control group by the end of experimental period. The GPT values of both groups of animals increased sharply during the first hour after release and maintained this increase throughout the rest of the observational period.

Total plasma SOD of control animals

Total plasma SOD activity of the control animals did not decrease until just prior to release of the tourniquet. The decrease was gradual and within 2 h reached a value approximately 10% lower than those of the controls. The SOD value declined steadily during the development of the shock state.

Discussions

Tourniquet shock has been employed as an experimental model of traumatic shock, and a number of investigations on the pathogenesis have been carried out [1–6]. The phenomenon of tourniquet shock, however, occurs

under relatively specific conditions. Ikeda et al. [6] pointed out the importance of the uniformity of the tourniquet application, and demonstrated a model in which rabbits were fastened by a hindlimb with a tourniquet used for infants. In the present study, we have attempted to define the conditions during tourniquet application by monitoring the pressure with a miniature sensor and an amplifier in the thighs of rats. This procedure caused 100% mortalitiy within 24 h of release in untreated rats. This experimental model, therefore, would be very useful to study tourniquet shock in respect to the reproducibility of the procedure and the induction of the shock states and mortality.

Tourniquet application and its removal provide not only physical tissue damage at the tourniquet site but also give rise to ischemia and reperfusion of the fastened hindlimbs. Ikeda et al. [6] mentioned that muscular tissue damage in the peripheral area of the tourniquet site played a important role in the induction of shock. Hiraiwa et al. [5] reported that the hypotension which occurrred in rabbits after removal of the tourniquet was due to a platelet activating factor generated in the reperfused hindlimb. These observations suggest that tourniquet shock has an aspect of ischemia-reperfusion injury which might sometimes be lethal [19].

It is now widely accepted that oxygen-free radicals have been implicated in the pathogenesis of severe tissue damage after ischemia-reperfusion in various organs [20–25] including skeletal muscles and skin of hindlimbs [26–28]. These highly reactive and toxic molecules are generated during purine nucleotide catabolism in tissue subjected to ischemia and reperfusion in the presence of xanthine oxidase [29, 30]. Hence, administration of free radical scavengers has been used to prevent tissue injury [9, 11, 21, 22, 27]. In a previous report [31], we indicated the protective effect of L-SOD, an enzymic scavenger, on the pathogenesis of tourniquet shock in rabbits.

In this experiment, the survival interval was prolonged by administration of L-SOD prior to tourniquet removal. In reperfused hindlimbs, vigorous peroxidation would occur, and the plasma SOD activity decreased promptly and continuously in the control animals. Prior saturation by administration of SOD would diminish the production of active oxygen in the reperfused hindlimbs. Rapid changes in the hematocrit as a result of increasing vascular permeability were improved by the pre-treatment in the early period of reoxidation. However, no significant differences were observed between the total plasma protein levels in the 2 groups of animals.

Local hyperoxidation would cause systemic hemodynamic and metabolic changes which might develop into circulatory shock. The increase of local vascular permeability would eventually lead to the hypoperfusion of other organs [4]. A number of studies including our previous report indicated that the oxygen-free radicals are also implicated in various circulatory shock mechanisms [7–11], and that SOD improved these pathological states [9, 11]. In the present experiment, however, it seems that the prior administration of L-SOD was not effective against the development of shock. Whereas L-SOD, which functioned locally as a scavenger in the early period after the reperfusion, contributed to a certain enhancement of the survival interval and rapid changes in blood proteins and enzymes.

Acknowledgment. The authors would like to thank Tosoh Co. for their kind gift of liposomal SOD and vacant liposome.

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