SUMMARY

Background. Ischemia/reperfusion (I/R) injury is one of causes of secondary expansion of tissue damage that can be occurred after a stoppage of the blood supply. I/R injury can be a serious problem related to use of tourniquet. There is few reports in which the influences of I/R injury for important organ using tourniquet are described.

Methods. Rats were subjected to bilateral hindlimb ischemia for 1, 2 or 3 hours followed by reperfusion for 4 hours. Levels of creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (Cre) and potassium ion as well as urinary protein were measured in all rats. Wet/dry ratios of lung and gastrocnemius muscle were used as an index of edema formation. Sections of gastrocnemius muscle and liver were analyzed histologically.

Results. We found that longer periods of ischemia were correlated with increased levels of CK, AST, ALT, BUN, and potassium ion as well as to the extent of muscle edema. AST, BUN and urinary protein levels after one hour or more of ischemia, CK, ALT levels after 2 hours or more of ischemia
and muscle edema after 3 hours of ischemia were significantly increased. With 3-hour ischemia, skeletal muscle neutrophils accumulated in blood vessels with some infiltrating the surrounding tissue, and the liver showed centrilobular congestion indicating dilatation of sinusoids around the central vein.

Conclusions. Limb ischemia for two hours or more is not recommended because local tissue is at risk and also because remote organs can be damaged.

Key Words: Ischemia/Reperfusion, Rat, Limb, Ischemia Period

INTRODUCTION

Ischemia/reperfusion (I/R) injury is a serious and occasionally fatal problem at the case of obstruction diseases. I/R injury not only affect local tissue but can also result in life-threatening damage to remote organs. Many researchers have investigated the biological/biochemical mechanisms of I/R injury as well as treatments.

In the field of orthopedics, I/R injury is a serious problem in cases of vascular disease, such as vascular disease caused by thrombosis of blood-supplying arteries to the lower extremities, free flaps and extended use of tourniquets. Surgeons usually limit tourniquet use to less than 1.5 hours, but its application may cause both local and systemic proinflammatory responses that can adversely affect patient outcome. In previous studies, I/R injury of the lower extremities was studied using an animal model, and various risk factors were revealed. Some suggestions on how to deal with I/R injury were also reported. A number of chemical and cellular mediators, including reactive oxygen species, cytokines (tumor necrosis factor-α, and interleukin-6), and neutrophils, have been implicated in the pathogenesis of I/R. Clinical and experimental studies have suggested that oxidative stress induced by reactive oxygen species is one of the most important mediators in this process. Deleterious oxygen radicals are produced in ischemic tissue. These are associated with lipid peroxidation and consequent disruption of cell membrane integrity, causing endothelial injury and increased microvascular permeability and tissue edema.

There have been various reports on experimental conditions for showing the effect of the ischemic period in rat limb I/R injury. However, there has been no study in which serum and urinary biochemical markers after various periods of ischemia induced by the use of a tourniquet were compared.

In this study using a rat model, changes in serum and urine biochemical markers with passage of time in the reperfusion phase after lower extremity ischemia were determined. The extent of damage to local skeletal muscle and remote organs of rats subjected to ischemia of 1, 2 or 3 hours and subsequent reperfusion was also determined.
MATERIALS AND METHODS

Animals

Thirty-six adult male Sprague-Dawley rats (Nihon SLC, Hamamatsu, Japan) weighing 330-380 g were used in this study. All of the rats were anesthetized preoperatively with intraperitoneal sodium pentobarbital (30 mg/kg). The rats were maintained under anesthesia throughout the procedure by additional intraperitoneal injections of pentobarbital (15 mg/kg) when required. Saline was continuously infused into the right jugular vein at a rate of 1.5 ml/kg/h.

The animals were randomly divided into 4 groups (n = 9 in each group): 3 I/R groups and a control group. In the I/R groups, bilateral hindlimb ischemia was induced through application of a non-loosening nylon cable tie (Daiso, Hiroshima, Japan) above the greater trochanter of each hindlimb as in previous studies. Ischemia was observed by skin color and coldness of the limbs and confirmed with a laser doppler blood perfusion monitor (FLO-N1, Omega wave, Tokyo, Japan). Rats were subjected to bilateral hindlimb ischemia for 1 hour (I/R), 2 hours (I/R) and 3 hours (I/R) followed by reperfusion for 4 hours.

Rats in the control group were subjected to sham hindlimb ischemia followed by sham reperfusion simply maintained under 5-7 hours of anesthesia with continuous saline infusion. This study was conducted according to the Guidelines for the Care and Use of Laboratory Animals of Nagoya City University Graduate School of Medical Sciences and was approved by the Experimental Protocol Committee of Nagoya City University Graduate School of Medical Sciences.

Serum and urinary biochemical markers for I/R injury

At the completion of the experiment, blood was removed from the abdominal aorta and urine was collected from the bladder by needle aspiration. Heparin was used as the anticoagulant for blood aspiration. Blood samples were centrifuged (3,000 g at 4°C for 10 min) to obtain serum. The amounts of creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (Cre) and potassium ion were measured. CK was measured with a JCA-BM8060 (Nihondenshi, Japan), AST and ALT were measured with a Hitachi7170 (Hitachi, Japan), BUN, Cre and K+ were measured with a Hitachi7180 (Hitachi, Japan) and urinary protein concentration was measured with a Hitachi7170 (Hitachi, Japan).

Wet/dry ratios of lung and gastrocnemius muscle

The right lung and gastrocnemius muscle were removed to weigh them for edema evaluation, and then they were dried at 100°C in a convection oven for 18h and reweighed (dry weight). Wet-to-dry ratios (W/D ratios) were calculated and used as an index of edema formation.
Histopathological analysis of various organs

The left lung, gastrocnemius muscle, kidney, and liver were removed and then fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at a thickness of 4 μm, affixed to glass slides, and stained with hematoxylin and eosin for histological analysis. The histological samples were examined by an expert pathologist for signs of inflammation.

Statistical analysis

The SPSS 14.0 for Windows was used for analyzing the data. The descriptive data were expressed as means ± SEM. One-way analysis of variance (ANOVA) was used for analyzing difference between groups. If the ANOVA was significant, the Tukey (if equality of variance was assumed) or Games-Howell (if equality of variance was not assumed) method was adopted as a post hoc test. All tests were two-sided. Differences were considered significant at P values less than 0.05.

RESULTS

Changes in biological/biochemical markers

At the completion of the study, 35 of the 36 rats were still alive: L/R (n = 8), L/R (n = 9), L/R (n = 9) and sham (n = 9). There were no significant differences in the markers studied among rats with 1, 2 and 3 hours of sham ischemia (data not shown), so we selected the rats with 2 + 4 hours continuous saline infusion as a control in the analysis. Hindlimb I/R resulted in a significant elevation of circulating levels of CK, ALT, potassium ion (2-h or 3-h ischemia), AST, BUN, and urinary protein (1-h, 2-h, or 3-h ischemia) compared to the levels in the sham groups (p < 0.05) (Fig.1. A-F). Between ischemia period, a significant elevation of ALT between 1-h and 2-h, CPK and BUN between 2-h and 3-h, AST and potassium ion among all ischemia groups was found (p < 0.05) (Fig.1. A-F). There was no significant elevation in the level of Cre compared to that in each of the sham groups (p > 0.05) (Fig.1. G).

Wet/dry ratios of lung and gastrocnemius muscle

Lung W/D ratios in the I/R groups showed no significant increase (p > 0.05) (Fig.2. A). Muscle W/D ratios in the I/R groups were significantly increased in the rats that were subjected to 3-hour ischemia comparing with the sham group and 2-hour ischemia (P < 0.05) (Fig.2. B).

Histopathological findings

In the L/R group, skeletal muscle showed marked accumulation of neutrophils in the blood vessels with some infiltration into the surrounding tissue (Fig.3. A). The liver showed centrilobular congestion indicating dilation of sinusoids around the central vein (Fig.3. B). Neutrophil infiltration was observed in all lung tissues. No remarkable change was observed in the kidney.
Changes in biological/biochemical markers. Hindlimb ischemia/reperfusion (I/R) resulted in a significant elevation of circulating levels of CK (A), ALT (C), potassium ion (D) (2-h or 3-h ischemia), AST (B), BUN (E), and urinary protein (F) (1-h, 2-h, or 3-h ischemia) compared to levels in the corresponding sham animals. The levels of CK, AST, ALT, BUN, and potassium ion in the I/R groups were correlated with the ischemic period. There was no significant elevation in Cre (G) compared to that in the sham animals. († P<0.05 versus sham, *P<0.05 I1/R versus I2/R, **P<0.05 I2/R versus I3/R)
DISCUSSION

The various orthopedic situations in which extremity ischemia can occur include arterial clamping during arterial reconstructive surgery, use of a tourniquet during a limb procedure and constriction of a limb under wreckage in a disaster. For recovery, blood reperfusion of the extremity is inevitably required. Although the pathophysiology of ischemia reperfusion injury is not fully understood, many previous reports suggest that reperfusion following prolonged ischemia stimulates excessive produc-
tion of reactive oxygen species (ROS) and triggers the participation of neutrophils causing an inflammatory reaction. In a normal environment, superoxide dismutase (SOD), which converts superoxide into hydrogen peroxide, acts to prevent superoxide from damaging tissue, but in reperfusion injury, these natural defenses may be overcome and hydrogen peroxide is converted into the hydroxyl radical, which can damage a wide variety of biological molecules including amino acids, membrane transport proteins and nucleic acids. The most important form of damage caused by oxygen free radicals is due to lipid peroxidation, which results in functional and structural cell alterations. The interpretation of the present results may be limited, because markers of oxidative stress were not measured in the present study.

The severity of local skeletal muscle injury has been assessed by measuring increases in CK and muscle edema. The cytosolic enzyme CK is found predominantly in muscle and is a reliable marker of muscle tissue damage. The present study showed that there were increases in plasma CK level and in muscle edema with prolongation of the ischemic period. Moreover, in rats that underwent 3 hours of ischemia, neutrophils accumulated in blood vessels of the hindlimbs and lung with some infiltrating the surrounding tissue. Lungs, liver and kidneys were examined to assess remote organ injury. Both AST and ALT, which have typically been used as markers of liver pathology, are also found in muscle and thus increases in their levels may be partly attributed to levels in muscle, rather than to liver damage alone. However, the histological finding of centrilobular congestion around the central vein in the liver with 3 hours of ischemia suggested that increases in these enzymes largely reflect liver injury. This study showed that liver function deteriorated due to limb ischemia reperfusion and that the extent of deterioration corresponded to the ischemic period. There have been several reports of lung injury induced by limb ischemia reperfusion, and the evaluation of lung function is important for determining clinical prognosis. In this study, however, there was no correlation between the sham and reperfused groups. The fact that extensive neutrophilic infiltration was observed not only in the I/R groups but also in some sham groups indicated that experimental methods, including intraperitoneal anesthesia, can affect the results.

In clinical studies, significant increases in levels of IL-1β, IL-6, and TNF-α has been found in patients who underwent elective knee arthroscopy or knee replacement and in whom a tourniquet was used. The present study indicated the possibility that reperfusion of the limb following even one hour of ischemia causes damage to not only local skeletal muscle but also to remote organs and that the extent of damage is dependent on length of the ischemic period. Ischemia of the lower limbs for two hours or more caused significant increases in levels of biochemical markers for muscle and liver injury, and histological changes were observed even after three hours. Ischemia for two hours or more is not recommended in rats since it may increase the risk of injury to both local tissue and remote organs. Similarly, in humans, ischemia of the lower limbs for two hours or more is not recommended even though the duration of operation can be longer than two hours.
REFERENCES

21. Vedder NB, Fouty BW, Winn RK, Harlan JM, Rice CL. Role of neutrophils in generalized reperfusion injury as-


