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Biochemical and histopathological changes in the mortality caused by acute ischemic limb injury: a rabbits' model

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Summary. Restoration of blood flow to an acute ischemic extremity may deteriorate the ischemic injury, lead to multiple organ dysfunction or even death. This paradox of continuing injury during reperfusion is not completely understood. The role of multi-organ damage in the mortality caused by ischemic limb injury is also still not clarified. The purpose of this study is to determine the biochemical and histopathological changes in the mortality caused by ischemic limb injury.

After anesthesia, the hindlimbs of 14 New Zealand white rabbits were made ischemic and set into 8 hours or 12 hours of ischemia. Blood samples were obtained then the creatine kinase (CK) levels were determined and CK isoenzymes analyzed. All rabbits with 8 hours' ischemia survived well, and 5 of the 7 rabbits with 12 hours' ischemia expired within 8 hours after reperfusion. CK elevation was correlated most strongly with the time of the ischemic insults. The percentage of CK-MB isoenzyme remained unchanged after 8 hours' ischemia-reperfusion insult, while increased significantly after 12 hours' ischemia-reperfusion insult. Histologic examinations showed that the major systemic manifestation was massive destruction of the liver and kidney. The injuries are more obvious in areas with the greatest blood flow during reperfusion. We concluded that the ratio of CK-MB isoenzyme is most useful for distinguishing the risk of mortality caused by acute ischemic limb injury, and the cause of systemic complications are attributed to the multi-organ failure.

Key words: Creatine kinase, Ischemic limb injury, Histopathology

Introduction

The restoration of blood supply after a period of ischemia not only permits survival of cells not killed by ischemia but also may result in further tissue injury (Walker, 1986). Although ischemic injury to skeletal muscle is of great clinical importance, relatively little is known about the systemic responses to the acute ischemic limb injury. Most experimental work has focused on myocardial ischemia; peripheral ischemia has been examined less frequently (Crillo et al., 1992). Indeed, cardiac ischemia during the perioperative period is the primary cause of mortality in most postoperative surgical patients (Mangano et al., 1990). Acute interruption of arterial blood flow to the extremities may also be associated with significant morbidity and mortality (Haimovici, 1979). Rhabdomyolysis, compartment syndrome, and even circulatory shock can result from skeletal muscle ischemia (Masten and Krugmire, 1978; Haimovici, 1979; Sjostrom et al., 1982). The CK-MB isoenzyme is found predominantly in cardiac tissue and thus has high specificity (Jaffe, 1990). In addition, there is high sensitivity; as little as 1 to 2 mg of tissue necrosis can be detected (Jaffe, 1990). In the clinical medicine, serial assays of creatine kinase (CK) and its isoenzyme CK-MB are the laboratory tests of choice for the diagnosis of myocardial injury (Galen, 1979; Grannis et al., 1979). Although muscle ischemia has been extensively studied in the heart, no studies have systematically characterized the cardiac response to the ischemic injury of skeletal muscle. The role of multi-organ damage in the mortality caused by ischemic limb injury is still not clarified. The purpose of this study is to elucidate the histopathological changes of multi-organs in the mortality caused by acute limb injury and the relation between the CK elevations and its clinical outcome.

Materials and methods

Fourteen New Zealand white rabbits weighing 2.5 to 3.0 kg were used in this study. The animals were fed Purina Laboratory Chow *ad libitum* and housed in a temperature-, humidity-, and light-controlled environment. Surgical procedures and experimental protocols were approved and under supervision by the Medical College's Animal Research Committee of the National Taiwan University. The rabbits were premedicated with

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0.5 mg atropine and then anesthetized by ketamine (25 mg/kg, s.c.) and Combelen® [N-(3'-dimethyl-amino-propyl)-3-propionylphenothiazine] (5 mg/kg, s.c., Bayer AG, Leverkusen, Engl.). The operative procedure was quite similar to that reported by Feller with some modification (Feller et al., 1989). Lower midline laparotomy was made. The right hindlimb was made ischemic by dissecting and double clamping the common iliac and external iliac artery with two microvascular clamps proximal and distal to the bifurcation of the internal iliac artery. Collateral flow to the ischemic leg was minimized by clipping the internal iliac, deep femoral, and iliolumbar arteries bilaterally and ligating both caudal abdominal and superficial epigastric arteries. In the pilot study, blockage of blood flow to the rabbits' lower leg was confirmed by disappearance of the internal iliac artery pulsation and the absence of active bleeding after amputation of toes. The right hindlimb of experimental rabbit was set into 8 hours or 12 hours of ischemia. Blood flow was restored to the limb at the end of the ischemic period by releasing the clamps and monitored by recovery of the pulsation of internal iliac artery. The animals were allowed to recover from anesthesia, placed into cages, and observed for mobidity and limb survival.

Sample preparation, CK determination and analysis of CK isoenzymes

For all animals, control blood samples were obtained with heparinized plastic syringes immediate before surgery. The vascular clamps were released after predetermined hours (8 or 12 hours) of ischemia. Series of blood samples were obtained at immediate after reperfusion, 1 hours, 2 hours, 24 hours, 7 days and 14 days of reperfusion. CK activity released from the cells into the whole blood is measured with a commercially available assay kit (procedure no. 47-UV, Sigma Co. USA). An aliquot (20 μl) from the plasma is mixed with 1 ml creatine kinase reagent, and the absorbance at 340 nm caused by NADH production is followed for 5 minutes at 30 °C. The change in rate of absorbance is directly proportional to creatine kinase activity. Another aliquot (75 μl) from the plasma was automatically analyzed by Helena® Rapid Electro-phoresis Analyzer for the percentage of each CK isoenzyme. The absolute values of each CK isoenzymes was then calculated by multiplying the total CK level with the percentage of each CK isoenzymes.

The differences between them were evaluated by a repeated measurement analysis of variances statistic method. The post hoc tests performed was Bonferroni's test. The level of statistical significance is defined as $P<0.05$.

Histopathological examination

Autopsy and necropsy of the experimental animals were performed immediately after their death. Multi-organs (including liver, spleen, kidney, heart, lung and brain) were dissected out and fixed with 4% formaldehyde in phosphate buffered solution for 18 hours, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Sections (5 to 7 μm in thickness) were cut and stained with hematoxylin and eosin and representative sections were photographed using high dry or oil immersion lens using light microscope. For comparison, two normal rabbits were also sacrificed at the completion of experiment simultaneously under sodium pentobarbital (25 mg/kg b.w.) anesthesia and served as a normal-controls.

Results

Biochemical analysis

The changes in the total CK levels of plasma were affected significantly by the ischemia-reperfusion insult. The changes of total CK and CK isoenzymes levels after 8 and 12 hours' ischemia-reperfusion insult were summarized in Tables 1 and 2. After 8 hrs' ischemic insult, the changes in total CK levels of plasma were affected significantly by the ischemia-reperfusion insult. There is statistically significant difference existed in these measurements ($P<0.00001$ by ANOVA test). When comparing with the control plasma sample, the CK levels increased significantly before 24 hours of reperfusion and decreased significantly after 7 days of reperfusion ($P<0.05$) (Table 1). After 12 hours of ischemic insult, the total CK levels of plasma also increased. When comparing with the control blood sample, the difference existed in these measurements is statistically significant ($P<0.00001$) (Table 2).

After 8 hrs' ischemic insult, the changes in CK isoenzyme levels of plasma were also affected significantly by the ischemia-reperfusion insult. The CK-BB levels increased significantly in the first two hours and then decreased; at 7 days after reperfusion, the CK-BB levels returned to the control level (Table 1). The CK-MB and CK-MM isoenzymes increased significantly in the first two hours and then decreased; at 7 days after reperfusion, the isoenzyme levels were decreased even lower to the control level (Table 1). Elevation of CK isoenzymes occurred without any definite clinical or EKG evidence of myocardial tissue damage and all the rabbits in this groups survived well. After 12 hrs' ischemic insult, the changes in CK isoenzyme levels of plasma were also affected significantly. The CK-BB, CK-MB, and CK-MM isoenzymes levels all increased significantly (Table 2).

During the initial two hours' reperfusion after ischemic insult, the percentage of CK-MB isoenzyme remained unchanged after 8 hours' ischemic insult, while increased significantly after 12 hours' ischemic insult (Fig. 1). On the contrary, the percentage of CK-MM isoenzyme increased significantly after 8 hours' ischemic insult; while there was no significant change after 12 hrs' ischemic insult (Fig. 2).

Histopathological examination

After a 12 hours' ischemia-reperfusion injury of the hindlimb, five of the seven rabbits (70%) died within the initial 8 hours' post-reperfusion period. Autopsy revealed that there were multiple organ changes. The liver is slightly enlarged, tense and mild congested with round edge. The kidney is normal or moderately increased in size. Immediately after the mortality caused by ischemic-reperfusion injury, morphological changes of heart, lung, spleen and skeletal muscle were not significant on gross examination.

Microscopically, the distributions of the cellular destruction were mainly at the liver, kidney and heart.

Liver

The histological change in the liver parenchyma is quite distinctive. There is massive necrosis with nearly complete removal of hepatocytes at the peri-portal area and the periphery of hepatic lobule (Fig. 3A). The necroses wipe out and destroying the peripheral area of the lobule. In the area of central vein, normal hepatic cells still existed. However, vacuolization of hepatic cells increased gradually from the central vein toward the periphery and portal area of the hepatic lobule. In the affected area, vacuolated hepatic cells with small vacuoles appeared within the cytoplasm were widely distributed (Fig. 3B,C). There are little or no inflammatory reaction in the viable margins.

Kidney

The glomerulus was congested with clump of red blood cells. The interstitium of the glomerulus was clumped with hyaline substance or even completely peeled off with hyaline cast at the center of the cavity (Fig. 4A). The most characteristic histopathological

Table 1. Changes of total creatine kinase (CK) and CK isoenzyme levels after 8 hours ischemica-reperfusion insult.

	TOTAL CK[a] (U/L)	CK-BB[b] (U/L)	CK-MB[c] (U/L)	CK-MM[c] (U/L)
Pre-ischemia	674.3±94.2 (403-897)	205.9±35.8 (89.5-344.3)	14.8±3.8 (0-29.5)	453.3±80.0 (259.1-721.7)
Immediate reperfusion	5943.3±176.2 (5405-6468)	1152.1±214.5 (416.6-2022.3)	108.4±21.6 (0-188.9)	4880.4±308.4 (3929.4-6524.0)
1 hr after reperfusion	8065.0±857.4 (5180-10610)	1405.7±324.8 (283.3-2667.0)	151.9±33.0 (0-246.4)	5908.4±784.7 (3589.4-8912.4)
2 hrs after reperfusion	9348.2±1550.7 (3370-14150)	155.3±415.2 (249.4-3734.3)	261.9±56.1 (87.6-541.0)	7678.3±1389.9 (3029.6-12848.2)
24 hrs after reperfusion	3518.6±431.8 (1809-5400)	610.0±116.8 (146.5-962.7)	74.8±18.5 (0-124.2)	3248.2±464.3 (1662.5-5375.6)
7 days after reperfusion	226.0±16.5 (173-293)	175.1±2.9 (0-19.7)	7.4±2.9 (0-19.7)	114.6±19.2 (46.8-196.3)
14 days after reperfusion	243.0±14.3 (203-303)	206.4±21.8 (142.7-307.4)	5.2±1.6 (0-10.6)	65.9±6.6 (51.75-97.0)
P value	< 0.00001	< 0.0001	< 0.00001	< 0.00001

[a]: the differences of the total CK levels at various time interval are all statistically significant to that of the control total CK level ($p<0.05$). [b]: when compared with the control plasma level, the differences of the CK-BB level before 7 days after reperfusion are statistically significant ($p<0.05$). [c]: the differences of the CK-MB and CK-MM levels at various time interval are all statistically significant to that of the control level ($p<0.05$).

Table 2. Changes of total creatine kinase (CK) and CK isoenzyme levels afer 12 hours ischemia-reperfusion insult.

	TOTAL CK (U/L)	CK-BB (U/L)	CK-MB (U/L)	CK-MM (U/L)
Pre-ischemia	780.6±100.5 (481-1193)	252.8±46.8 (138.0-492.8)	16.8±4.0 (0-34.4)	511.1±64.4 (330-830.3)
Immediate reperfusion	14557.7±2070.4 (5454-20673)	2127.0±337.7 (1150.8-3488.2)	2817.8±462.4 (627.2-4093.3)	9612.3±1698.3 (3670.5-16369.7)
1 hr after reperfusion	43741.0±9459.4 (14610-69187)	5600.4±1030.8 (2220.7-9387.9)	8004.4±1714.9 (2702.9-14343.3)	30409.0±7758.6 (8310.1-59846.8)
2 hrs after reperfusion	56433.0±4675.9 (42103-70766)	9215.1±899.8 (6224.7-12337.5)	14357.2±1826.9 (10415.0-23423.6)	32860.0±2891.0 (23956.6-46045.4)
P value	< 0.0001	< 0.00001	< 0.00001	< 0.00001

After 12 hours of ischemia, the changes in total CK, CK-BB, CK-MB, and CK-MM isoenzymes levels all increases signfiicantly to that of control level ($p<0.05$).

changes include marked cellular swelling (hydropic degeneration) of renal tubular cells adjacent to the glomerulus. The degenerated cells appeared vaculoated and contained displaced dark nuclei (Fig. 4B). The tubular epithelia of the renal medulla are relatively well preserved after this ischemic-reperfusion insult.

Heart

Microscopically, small foci of dark coagulated myofibers with preserved outlines contrast with surrounding viable normal myocardial cells were quite obvious (Fig. 5A). Simultaneously, wavy fibers showing coagulative changes, elongation, and narrowing were also visible (Fig. 5B). There is fragmentation of the cardiac fibers contain hemorrhage with stagnated and trapped blood (Fig. 5C)

Lung

Microscopically, some of the alveoli are collapsed, others still distended (Fig. 6A). There is some inflammatory cells infiltration within the respiratory tract (Fig. 6B).

Spleen, brain and skeletal muscle

Spleen, brain and skeletal muscle did not show any significant histological changes as compared to those in normal-control animals.

Discussion

Restoration of blood flow to acute ischemic lower limb muscles generates cellular and biochemical mediators that may initiate a cascade of pathologic events, ultimately leading to the development of the multi-organ failure and high mortality rate (Haimovici, 1960; McCord, 1985; Ali et al., 1993; Pang et al., 1993). The management of an acute ischemic injury of the lower extremity requires close observation for the development of complications. Both local (compressive muscle necrosis) and systemic renal and myocardial failure (Labbe et al., 1987), multiple organ dysfunction, or even death may occur. This paradox of continuing injury during reperfusion is incompletely understood and most likely multi-factorial in etiology (Blaisdell, 1989). The purpose of this study is to evaluate the biochemical and morphological changes of multi-organs to the mortality caused by acute ischemic injury of skeletal muscle.

Skeletal muscle is relatively resistant to ischemia for up to 2 hr with minimal risk of ischemic necrosis (Rutherford, 1987; Eckert and Schnackerrz, 1991). With prolonged periods of ischemia, total muscle necrosis can result. However, there are many clinical situations in which skeletal muscles are subjected to warm global ischemia, such as free flap transfer, replantation of amputated limbs, post-traumatic compartment syndrome, application of vascular clamps or tourniquets in reconstructive surgery, embolism, and thrombosis (Pang et al., 1993). The duration of ischemia is considered to be the most important factor determining the outcome; above 6 hours of ischemia, the incidence of death, limb loss, or both increases (Kendrick et al., 1981). In this study, after 8 hours of ischemia and reperfusion, elevation in total CK level was found. The main cause of increased in total CK level was due to the marked efflux of CK-MM isoenzyme from the muscular tissue (Table 1). After 7 days' reperfusion, the total CK level (including the CK-MB and CK-MM levels) declined to a level even lower than the control samples. Similar results were also noted in the work of McArdle et al. (1994). The possible explanation is that the regenerating

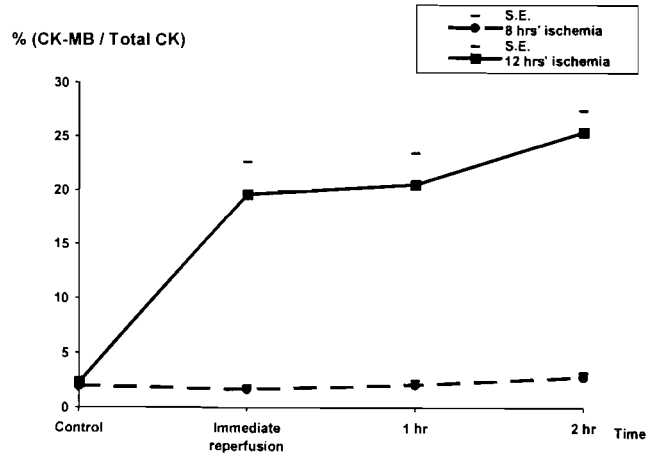

Fig. 1. Changes of the percentage of CK-MB isoenzyme (100% x CK-MB / total CK) in the initial 2 hours of reperfusion after 8 and 12 hours' ischemic insult. The changes in the percentage of CK-MB isoenzymes of blood were increased significantly after 12 hours' ischemia-reperfusion insult. On the contrary, the percentage of CK-MB isoenzyme remained unchanged after 8 hours' ischemia-reperfusion insult.

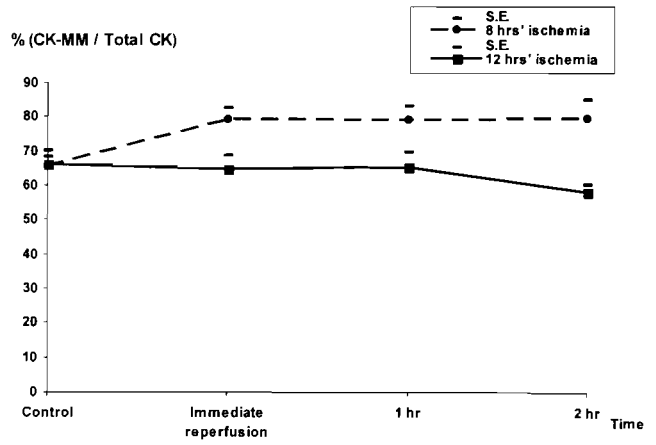

Fig. 2. Changes of the percentage of CK-MM isoenzyme (100% x CK-MM / total CK) in the initial 2 hours of reperfusion after 8 and 12 hours' ischemic insult. The percentage of CK-MM isoenzyme increased significantly after 8 hours' ischemia-reperfusion insult; while there is no significant change in the percentage of CK-MM isoenzyme after 12 hours' ischemia-reperfusion insult.

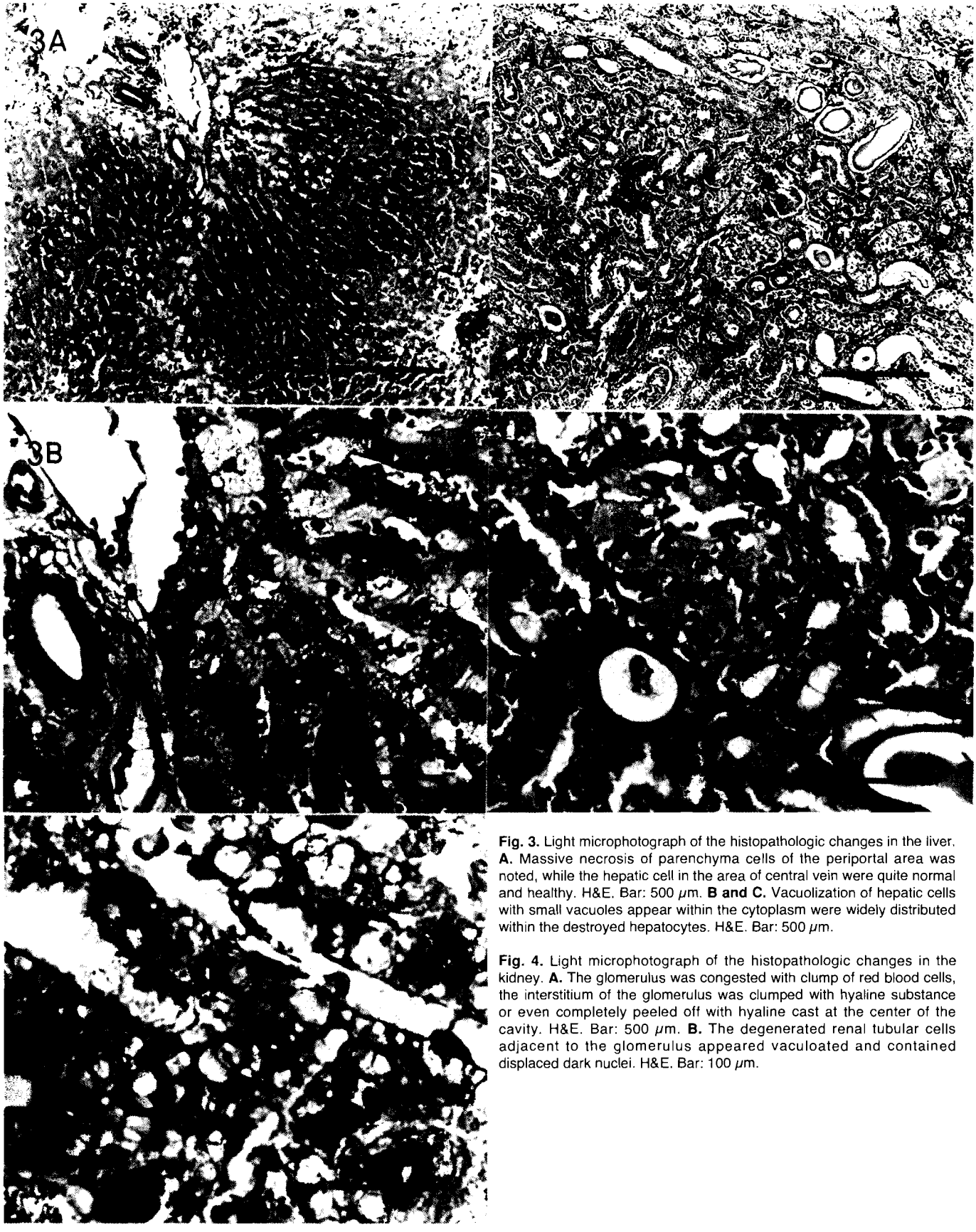

Fig. 3. Light microphotograph of the histopathologic changes in the liver. **A.** Massive necrosis of parenchyma cells of the periportal area was noted, while the hepatic cell in the area of central vein were quite normal and healthy. H&E. Bar: 500 μm. **B and C.** Vacuolization of hepatic cells with small vacuoles appear within the cytoplasm were widely distributed within the destroyed hepatocytes. H&E. Bar: 500 μm.

Fig. 4. Light microphotograph of the histopathologic changes in the kidney. **A.** The glomerulus was congested with clump of red blood cells, the interstitium of the glomerulus was clumped with hyaline substance or even completely peeled off with hyaline cast at the center of the cavity. H&E. Bar: 500 μm. **B.** The degenerated renal tubular cells adjacent to the glomerulus appeared vaculoated and contained displaced dark nuclei. H&E. Bar: 100 μm.

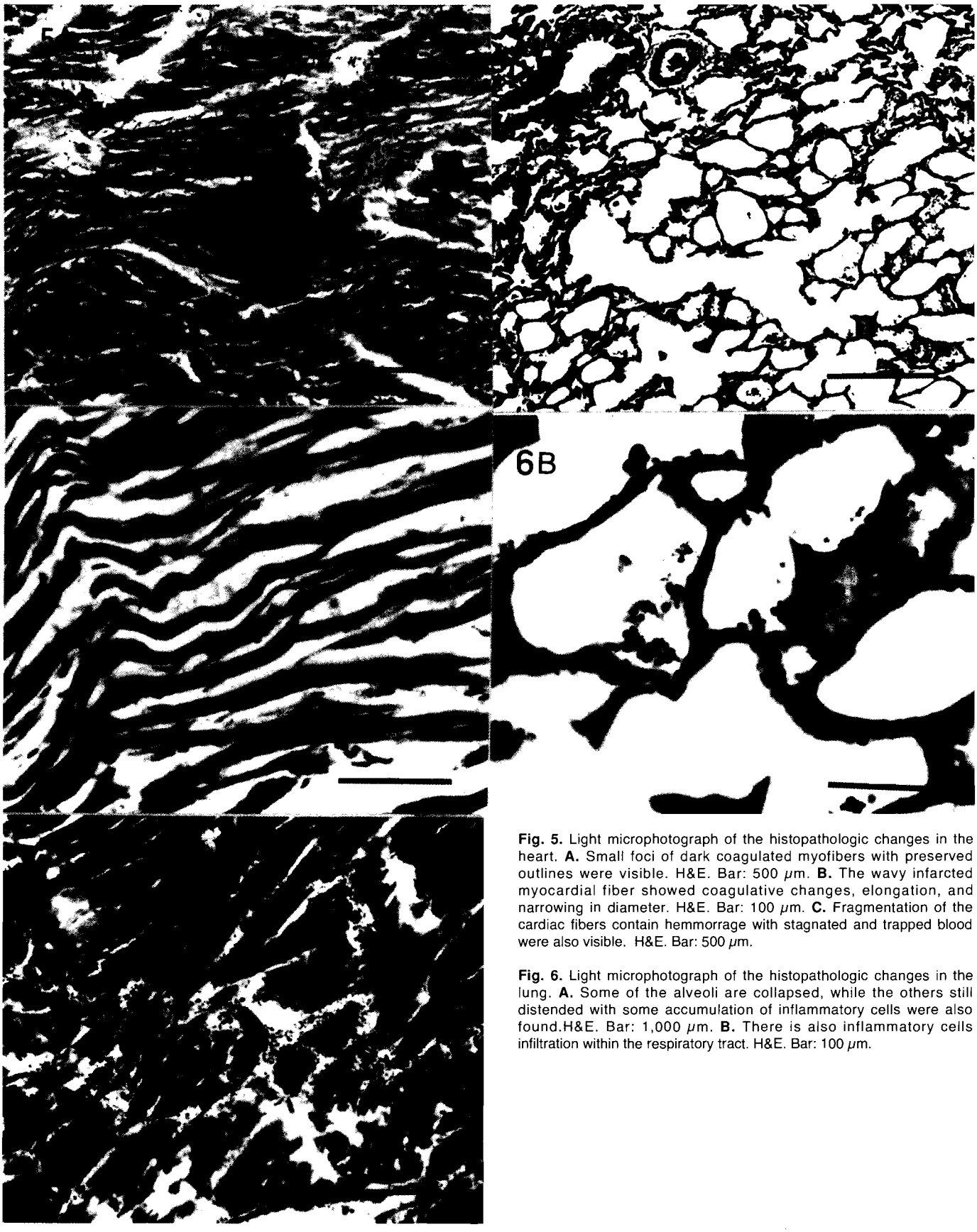

Fig. 5. Light microphotograph of the histopathologic changes in the heart. **A.** Small foci of dark coagulated myofibers with preserved outlines were visible. H&E. Bar: 500 μm. **B.** The wavy infarcted myocardial fiber showed coagulative changes, elongation, and narrowing in diameter. H&E. Bar: 100 μm. **C.** Fragmentation of the cardiac fibers contain hemmorrage with stagnated and trapped blood were also visible. H&E. Bar: 500 μm.

Fig. 6. Light microphotograph of the histopathologic changes in the lung. **A.** Some of the alveoli are collapsed, while the others still distended with some accumulation of inflammatory cells were also found.H&E. Bar: 1,000 μm. **B.** There is also inflammatory cells infiltration within the respiratory tract. H&E. Bar: 100 μm.

muscles contained a lower total CK activity than the control muscles, which accounted for the reduced CK efflux after experimental damage (McArdle et al., 1994).

The changes in the total CK levels were affected significantly by length of the ischemic insult. Elevations were even higher after surgery that employed a 12 hours' ischemic insult (Table 2). All rabbits survived after 8 hrs ischemic insults; while five of 7 rabbits after 12 hours' ischemia expired during the first 8 hours' reperfusion period. The changes in the CK isoenzymes were also affected significantly by length of the ischemic time. After 8 hours' ischemia, the absolute level of CK-MB isoenzyme increased significantly especially during the first 24 hours but much lower than that with 12 hours' ischemic insult (Tables 1, 2).

Elevations of total CK and CK-MB have been observed in various disease in the apparent absence of evidence of myocardial injury (Healey et al., 1985; Wukich et al., 1990; Burnett et al., 1994; Lenke et al., 1994). Small amounts of CK-MB were also found in skeletal muscle (Goto et al., 1969; Lamplugh et al., 1979). These small amounts are rarely problematic, but increases in CK-MB after acute and/or chronic skeletal muscle injury can occur, raising the amount of CK-MB in the blood sample (Lenke et al., 1994). Perhaps for this reason, elevations of total CK and CK-MB have been observed after ischemic limb injury (Tables 1, 2).

In acute myocardial injury, the CK-MB begins to rise as early as 6 h with peak values occurring at 18 to 20 h (Jaffe, 1990). This rising and falling pattern has been generally advocated to distinguish skeletal muscle release of CK-MB from cardiac release. Obviously, enzymes alone should not be used to diagnose acute myocardial infarction in this or any other setting. In the group with 8 hours' ischemia, the percentage of CK-MB/total CK was remained unchanged (Fig. 1); while the percentage of CK-MM/total CK was increased significantly (Fig. 2); and all the rabbits in this group survived the postoperative period. One possibility should be considered to explain the increased levels of CK-MB after ischemic insults. The level of CK-MB increased after 8 hours' ischemia is due to the small amounts of MB found in skeletal muscle(Goto et al., 1969; Lamplugh et al., 1979). The lack of clinical or EKG data of infarction argues strongly that release was from skeletal muscle. On the other side, marked elevation of the CK-MB level (Table 2) with marked elevation in percentage of CK-MB/ total CK (Fig. 1) were noted after 12 hours' ischemic insult; and most rabbits in this group (5 out of 7 in this study) died postoperately. This facts strongly supports the possibility of the cardiac origin of the CK-MB isoenzyme in the rabbits died after 12 hours' ischemia.

As noted above, the duration of ischemia is considered by many to be the most important clinical factor determining the outcome. Above a (grace period) of 6 hours of ischemia, the incidence of death, limb loss, or both increased (Kendrick et al., 1981). Deleterious systemic effects resulting from reperfusion of acutely ischemic extremities were recognized several decades ago by Haimovici (1960, 1979). He applied the term «myopathic-nephrotic-metabolic syndrome» to describe the acute metabolic abnormalities associated with myoglobinuria and kidney failure after acute limb ischemia. More recently, experimental works have demonstrated that reperfusion of ischemic limbs produces significant injury to distant organs, including heart and lungs (Klausner et al., 1988; Howard et al., 1991; Lindsay et al., 1991). However, the injury on the liver had not been reported. In this study, after a 12 hours' ischemia-reperfusion injury of a hindlimb, five of the seven rabbits died within eight hours post-reperfusion period. The major systemic manifestation was massive destruction of multi-organs. The cellular destructions were mainly distributed at the liver, kidney and the heart.

Microscopically, the destruction of liver parenchyma is actually located at the periphery of the arterial and portal supply (Figs. 3). This is quite different from that occurs with shock or cardiac failure, which located at the area around of the central vein (Arcidi et al., 1981). In the kidney, cellular necrosis occurred mainly in the tubular epithelial cells of the renal cortex (Fig. 4). The tubular epithelium of the renal medulla is relatively well preserved. Both the cellular injuries of liver and kidney occurred at input of systemic blood supply. In the mortality caused by ischemic-reperfusion injury, there are small foci of dark coagulated myofibrils and fragmentation of myofibrils accompanied by hemorrhage (Fig. 5). It is thought that these changes result from the forceful systolic tugs by the viable fibers immediately adjacent to the non-contractile dead fibers, stretching and buckling them (Cotran et al., 1989).

Changes in the pulmonary parenchyma and microvasculature following ischemia of the lower extremities have been described (Stallone et al., 1969; Seekamp et al., 1993). Pulmonary hypertension and leukosequestration following lower torso ischemia in a canine model (Anner et al., 1987), and similar changes following hypovolemic shock have been reported (Redl et al., 1984). In this study, the pulmonary parenchyma showed scattering of collapsed and distended alveoli containing red cells and some inflammatory cells (Fig. 6). We thought that in the toxicity induced by reperfusion, the injurious agents may directly affect cell membranes of the endothelium, and subsequently to increase permeability, and induce the leakage and accumulation of red cells and inflammatory cells within the alveolae.

The mechanism by which organ damage produced by prolonged anoxia or ischemia are still not completely understood. In the previous work of our institute, oxygen-derived free radicals had been suggested to play a causative role in the pathology of reperfusion injury (Sun et al., 1996). The basis for tissue injury during ischemia depends on depletion of tissue oxygen and energy substrates. Following acute arterial occlusion, the restoration of blood flow heralds the onset of

biochemical events, forming the basis of what is known as the reperfusion syndrome. As a result, this tissue injury is maximal in areas with the greatest blood flow during reperfusion (Sabido et al., 1994).

In summary, we concluded that enzyme elevations should not be used in isolation to diagnose myocardial injury in the patients of the ischemic limb injury; and the CK-MB isoenzyme ratio is useful for distinguishing the risk of mortality after ischemic limb injury. In the mortality after acute ischemic limb injury, the causes of systemic complications are mainly attributed to the multi-organ failure. In the histopathological examination, the tissue injury is maximal in areas with the greatest blood flow during reperfusion. This is in consistent to the hypothesis that the oxygen-derived free radicals play a causative role in the pathology of reperfusion injury. Further studies are required to clarify these biochemical mechanisms at the cellular and molecular basis.

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